Constitutive activation of phosphoinositide 3-kinase (PI3K) stimulates glucose transport and GLUT4 glucose transporter translocation to the plasma membrane in adipocytes. To determine whether a direct interaction of PI3K with GLUT4-containing vesicles (hereafter called GLUT4 vesicles) is important for the effect of insulin on GLUT4 translocation, we targeted constitutively active PI3K to GLUT4 vesicles. We fused the inter-Src homology region 2 of the regulatory p85 subunit of PI3K (iSH2) either to a C-terminal sequence of GLUT4 (G4c, amino acids 406–509) or to this region and the N-terminal tail of GLUT4 (G4a, amino acids 1–19), resulting in the fusion proteins iSH2-G4a and G4a-iSH2-G4c, respectively. Coexpression of the fusion proteins or untargeted iSH2 with the catalytic p110α subunit of PI3K (p110) in 3T3-L1 adipocytes by adenovirus-mediated gene transfer increased total PI3K activity in homogenates 11–13-fold over nontransduced cells or cells transduced with adenovirus encoding β-galactosidase. In contrast, PI3K activity in GLUT4 vesicles increased 11- to 13-fold with expression of either targeted construct and p110 but only 2-fold with the untargeted iSH2 and p110, indicating successful targeting of PI3K to GLUT4 vesicles. Both targeted and nontargeted constructs stimulated DNA synthesis to levels greater than insulin, demonstrating that both types of constructs had biologic activity in intact cells. Despite this, untargeted iSH2/p110 coexpression was more effective in stimulating 2-deoxyglucose uptake (6-fold) than either iSH2-G4c/p110 or G4a-iSH2-G4c/p110 coexpression (both 2-fold). Only iSH2/p110 coexpression led to a significant GLUT4 translocation to the plasma membrane. Insulin-stimulated glucose transport was unaffected by any construct. Thus, a direct interaction between PI3K and GLUT4 vesicles is either not required or not sufficient for GLUT4 translocation and stimulation of glucose transport.

Activation of phosphoinositide 3-kinase (PI3K) plays a central role in the insulin-signaling cascades leading to metabolic as well as mitogenic cellular responses. Studies using either inhibitors such as wortmannin and LY294002 or dominant negative forms of PI3K have demonstrated that PI3K activation is necessary for many of the effects of insulin (1–3). In addition, more recent studies demonstrate that increased PI3K activity is at least partially sufficient to stimulate GLUT4 translocation, glucose transport, DNA synthesis, and actin reorganization in 3T3-L1 adipocytes in the absence of insulin stimulation (4–7). Other growth factors such as platelet-derived growth factor (PDGF) as well as cytokines activate PI3K, but they do not stimulate the same metabolic effects as insulin (8, 9). Thus, the mechanism(s) underlying the specificity of the effects of insulin is of great interest. This specificity might be explained by a distinct spatial distribution of the signaling events induced by different growth factors and cytokines. For example, while PDGF-stimulated PI3K activity resides primarily in the plasma membrane (PM), insulin-stimulated PI3K activity is found primarily in an intracellular membrane compartment, the “low density” microsomes (LDM) (10–13), and is associated with insulin-responsive substrates (IRSs).

The LDM compartment consists of a wide range of membrane vesicles, up to ~35% of which contain the insulin-responsive glucose transporter, GLUT4 (14). Following insulin stimulation, GLUT4 is redistributed from the LDM to the plasma membrane, resulting in increased glucose transport (15). The exact molecular mechanisms that result in translocation of GLUT4 vesicles are poorly understood. However, they are the subject of intense research, since there are no alterations in GLUT4 primary structure or protein expression in skeletal muscle of humans with obesity or type II diabetes, and evidence indicates that a defect in GLUT4-vesicle translocation and/or fusion with the PM may be a major factor underlying the insulin resistance associated with these states (15–17). Hence, manipulating insulin signaling pathways might lead to new therapeutic approaches to overcome defective GLUT4 vesicle translocation/fusion and thus prevent or ameliorate insulin resistance.

A compelling hypothesis for the mechanism of GLUT4 vesicle translocation is that PI3K activity associates directly with the GLUT4 vesicle, leading to translocation, possibly either by recruitment of downstream signaling molecules or other proteins or by generation of the highly charged phospholipid products of PI3K: phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. A recent report indicates that insulin stimulates translocation of IRS-1 to the GLUT4 vesicle and that this is associated with stimulation of PI3K in DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; IRS, insulin-responsive substrate; pfu, plaque-forming units; TBS, Tris-buffered saline.

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the GLUT4 vesicle itself (18). Other reports do not find colocalization of the majority of IRS-1 or the p85 regulatory subunit of PI3K with GLUT4 (10, 19, 20), although it is still possible that a small amount of IRS-1 and p85 could associate with the GLUT4 vesicle or that the association was missed due to its transient nature or to experimental methodologies, which cause dissociation of these proteins from the vesicles.

Thus, we took a novel approach to test the hypothesis that the compartmentalization of PI3K activity in GLUT4 vesicles will augment GLUT4 translocation and glucose transport. We recently found that coexpression of the inter Src homology region 2 of the P85α subunit of PI3K (iSH2) with the catalytic p110α subunit of PI3K (p110) by adenovirus-mediated gene delivery in 3T3-L1 adipocytes stimulates PI3K activity in the LDM and cytosol to levels above insulin stimulation and increases GLUT4 translocation and glucose transport to ~40% of the effect of insulin (4). In the current study, we targeted PI3K activity to GLUT4 vesicles to determine whether this would give the 15–20-fold stimulation of glucose transport seen with insulin (4).

Since both the N-terminal and C-terminal domains of GLUT4 have been shown to play a role in the intracellular trafficking and targeting of GLUT4 (21–24), we fused to iSH2 either the two C-terminal membrane-spanning domains and the cytosolic C-terminal tail of GLUT4 (G4c, amino acids 406–509) or this region and in addition the N-terminal tail of GLUT4 (G4n, amino acids 1–19). This resulted in the fusion proteins iSH2-G4c, G4n-iSH2-G4c, respectively. Coexpression of these fusion proteins with p110 in 3T3-L1 adipocytes results in a dramatic increase of PI3K activity in GLUT4-containing vesicle as well as stimulation of DNA synthesis. However, the targeted expression of PI3K is less efficient in stimulating GLUT4 translocation and glucose transport than untargeted expression of PI3K. These results indicate that an increase in GLUT4 vesicle-associated PI3K activity is not required or not sufficient to stimulate GLUT4 translocation. The unique ability of insulin-stimulated PI3K activity to trigger this translocation may be linked to localization in a different membrane subfraction of the LDM or may not require membrane association.

EXPERIMENTAL PROCEDURES

**Cell Culture and Transfections—**3T3-L1 cells (ATCC CCL 92.1, Rockville, MD) were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies, Gaithersburg, MD) at 37 °C, 5% CO2. Two days after confluence, differentiation was induced with 0.5 mm 3-isobutyl-1-methylxanthine, 0.25 μg/ml dexamethasone (both from Sigma) and 1 μg/ml insulin (porcine, crystalline, gift of R. Chance, Eli Lilly) for 3 days. During and following differentiation, DMEM was supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA) instead of calf serum. Cells were used for experiments 10–20 days after the second spin was resuspended in HES, layered on a 1.12M sucrose cushion, and centrifuged at 100,000 × g for 75 min to yield total membranes (pellet) and cytosol (supernatant). PM, high density microsome, and LDM fraction as the supernatant. PM, high density microsome, and LDM

**Plasmids—**The plasmid pCMV-iSH2 containing the Myc tag sequence fused to the carboxy-terminal sequence of the iSH2 region (amino acids 428–678) of the human p85α subunit of PI3K (27) was provided by T. F. Franke (Columbia University, New York). The coding region was removed from this plasmid and cloned into pACCMV.pLPA (28), provided by C. Newgard (University of Texas Southwestern Medical Center, Dallas, TX), resulting in the plasmid pACCMV.pLPA-iSH2 as described before (4). A plasmid encoding a hemagglutinin tag fused to the amino-terminal sequence of the iSH2 region was provided by K. Auger (Dana Farber Cancer Institute, Boston, MA). An EcoRI–BspHI fragment of this plasmid encoding the hemagglutinin tag and iSH2 region was ligated in frame to a polymerase chain reaction-generated BspHI–SalI fragment encoding the full-length GLUT4 (amino acids 406–509) and cloned into pACCMV.pLPA, resulting in the plasmid pACCMV.pLPA-iSH2-G4c. A polymerase chain reaction-generated fragment encoding amino acids 1–19 of the human GLUT4 was cloned in frame to the hemagglutinin tag encoding 5′-end of this fusion construct to generate the plasmid pACCMV.pLPA-G4n-iSH2-G4c. The full-length proteins were sequenced in the regions derived by polymerase chain reaction using standard techniques. The plasmid pcG-110 containing a constitutively active PI3K was provided by A. Klippe (University of California, San Francisco, CA) (29). The Myc-tagged mouse p110 subunit of PI3K was amplified from this plasmid by polymerase chain reaction and cloned into pACCMV.pLPA, resulting in the plasmid pACCMV.pLPA-110 as described before (4).

**Generation of Recombinant Adenovirus and Transduction of 3T3-L1 Adipocytes—**Recombinant adenoviruses were generated as described by Becker et al. (26). Briefly, the plasmids pACCMV.pLPA-iSH2, pACCMV.pLPA-iSH2-G4c, pACCMV.pLPA-G4n-iSH2-G4c, and pACCMV.pLPA-110 were each cotransfected with the plasmid pJM-17 into 293 cells as described above. Cell lysis indicating a recombination event, occurrence of cotransfection. Several recombinant virus were checked for the successful integration of the respective coding regions by Western blotting using lysate of transfected 293 cells. One clone for each of the constructs was amplified further in 293 cells. Purification of virus from 30–40 15-cm diameter plates by cesium chloride centrifugation resulted in high titer stocks of recombinant virus, typically 1–2 × 1010 pfu/ml as determined by limiting dilution. The recombinant adenovirus encoding β-galactosidase was provided by C. Newgard and amplified as described above.

**Transduction of Differentiated 3T3-L1 Adipocytes—**Differentiated 3T3-L1 adipocytes were performed overnight with constant agitation on a rocking platform in DMEM with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies, Gaithersburg, MD) at 37 °C, 5% CO2. Two days after confluence, differentiation was induced with 0.5 mm 3-isobutyl-1-methylxanthine, 0.25 μg/ml dexamethasone (both from Sigma) and 1 μg/ml insulin (porcine, crystalline, gift of R. Chance, Eli Lilly) for 3 days. During and following differentiation, DMEM was supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA) instead of calf serum. Cells were used for experiments 10–20 days after the second spin was resuspended in HES, layered on a 1.12M sucrose cushion, and centrifuged at 100,000 × g for 75 min to yield total membranes (pellet) and cytosol (supernatant). PI3K activity was measured at 1 × 109 pfu/ml each, resulting in a total of 2 × 109 pfu/ml in cotransduction experiments. Cotransduction with two viruses (each at 109 pfu/ml) simultaneously did not reduce either the percentage of cells transduced or the level of expression compared with one virus alone (4).

**Cell Fractionation—**To generate a total membrane fraction and a cytosolic fraction, COS-7 or 3T3-L1 cells were incubated in DMEM with 0.1% calf serum for 18 h, stimulated or not with 100 nM insulin for the indicated time, homogenized by 20 strokes in a Potter homogenizer in PI3K lysis buffer (see below), and centrifuged at 180,000 × g for 75 min to yield total membranes (pellet) and cytosol (supernatant). In separate experiments, representation was performed by 20 strokes in a Potter homogenizer and centrifuged at 19,000 × g for 20 min. The pellet of this first spin was resuspended in HES and centrifuged again under the same conditions. The resulting pellet from this second spin was resuspended in HES, layered on a 1.12 μm sucrose cushion, and centrifuged at 100,000 × g for 30 min. The resulting interphase was collected, washed in HES, and spun at 50,000 × g for 30 min to yield the PM fraction. The supernatant of the first spin was centrifuged at 41,000 × g for 20 min. The resulting pellet was the high density microsome fraction, and the resulting supernatant was spun at 180,000 × g for 75 min to yield the low density microsome fraction (LDM) as the pellet and the cytosolic fraction as the supernatant. PM, high density microsome, and LDM fractions were resuspended in equal volumes of PI3K lysis buffer. This fractionation procedure had previously been characterized (4). Protein recoveries (μg/100-mm diameter well) and purity of membrane fractions were not altered by transduction with recombinant adenoviruses.

**GLUT4 Vesicle Preparation—**GLUT4 vesicles were prepared as described previously (30). Following incubation in DMEM containing 0.1% CS overnight, differentiated 3T3-L1 adipocytes were or were not stimulated with 100 nM insulin for the indicated times, quickly washed with

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PBS, and placed on ice. The cells from one 100 mm diameter plate were scraped into 3.0 ml of ice-cold homogenization buffer (150 mM KCl, 2 mM MgCl2, 20 mM HEPES, 1 mM sodium orthovanadate, 2 μM/ml aprotinin, and leupeptin, pH 7.2) and homogenized using 20 strokes of a Potter homogenizer. Following a 20-min spin at 40,000 × g, the supernatant (LDM/cytosol) was split into two tubes for immunoadsorption with either affinity-purified anti-vp165 antibody (30) or equal amounts of irrelevant rabbit IgG (Sigma), both bound to protein A on the surface of formaldehyde-fixed Staphylococcus aureus cells (10 μg of antibodies on 4 μl of S. aureus cells). After incubation for 2 h on a rotating wheel at 4 °C, the S. aureus cells were collected by centrifugation and washed three times in homogenization buffer and split into two tubes. One half was resuspended in 25 μl of PI3K lysis buffer (see below) and immediately used for the determination of PI3K activity; the other half was incubated in PI3K lysis buffer with 1% Nonidet P-40 for 1 h on ice, and the solubilized proteins were used for Western blotting.

Glucose Transport—Glucose transport activity was determined as described (31). 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 minimal essential medium. 2-[3H]Deoxy-D-glucose (0.33 Ci/35-mm-diameter well; NEN Life Science Products) 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 (v/v) ice-cold phloretin solution (82 mg/liter in PBS). Cells were then 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 described previously (25).

PI3K Assay—Total PI3K activity in membrane and cytosolic fractions was assayed as described for cytosol (32), using the borate thin layer chromatography method described by Walsh et al. (33), which allows separation of phosphatidylinositol 5-phosphate and phosphatidylinositol 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, 10% glycerol (pH 7.4), containing 1 mM sodium orthovanadate, 2 μg/ml aprotinin and leupeptin, and 0.5 mM dithiothreitol). Cytosol and total membranes were prepared as described under “Cell fractionation.” For determination of PI3K activity in EM and LDM fractions, cells were homogenized and fractionated in HES buffer as described above, and the fractions were resuspended in PI3K lysis buffer. For determination of PI3K activity in GLUT4 vesicles, vesicles were adsorbed to S. aureus cells, washed as described above, and resuspended in 25 μl of 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/ml phosphatidylinositol, 80 μM ATP, 0.8 μCi/μl [γ-32P]ATP (3000 Ci/mmol; NEN Life Science Products), 20 mM HEPES (pH 7.5), 50 mM NaCl, 12.5 mM MgCl2, and 0.015% Nonidet P-40. To inhibit some of the phosphoinositide 4-kinase activity in membrane fractions, adenosine was added to be equal to a final concentration of 200 μM (34). The reaction was stopped after 5 min by the addition of 90 μl of 1% HCl and the phospholipids were extracted with 160 μl of chloroform/methanol (1:1, v/v). Phosphatidylinositol monophosphate in 40 μl organic phase was separated by borate thin layer chromatography on aluminum-backed Silica Gel 60 plates (EM Separations, Inc., Gibbstown, NJ; pretreated with a solution containing 25 mM 2-amino-5-dimethylamino-1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid (CDTA, Sigma), 50% (v/v) ethanol, and 0.06% NaOH and then dried for 1 h and baked at 110 °C for 15 min) in developing solution containing 37.5% (v/v) methanol, 30% (v/v) chloroform, 22.5% (v/v) pyridine (Sigma), 1.33% (v/v) formic acid, 1 N boric acid, and 8.5 mM butylated hydroxytoluene (Sigma) and detected by autoradiography. Quantification of phosphatidylinositol 3-phosphate was performed using a PhosphorImager (Molecular Dynamics, Inc., model 425E). Under the conditions described above, activity was linear with time and proportional to protein concentration.

Western Blotting—Polyacrylamide gel electrophoresis and Western blotting was performed as described previously (35, 36) using 1.5-mm thick minigels (Novex, San Diego, CA), nitrocellulose membranes (0.45 μm, Schleicher & Schuell), and the Mini Trans-Blot Transfer cell (BioRad). Bovine serum albumin buffer, 20% (v/v) methanol. Following incubation in Tris-buffered saline (TBS), 0.05% (v/v) Tween 20, 5% (v/v) low fat dry milk for 2 h, the membranes were incubated with the primary antibody overnight (polyclonal anti-GLUT4 (C-terminal) provided by H. Haspel (Henry Ford Health Center, Detroit, MI), 1:400; polyclonal anti-GLUT4 (N-terminal) provided by G. Gould (University of Aberdeen, Scotland), 1:400). Membranes were washed in TBS, 0.05% Tween 20 (anti-N-terminal GLUT4) or TBS, 0.5% Tween 20 (anti-C-terminal GLUT4) for 15 min; incubated with horseradish peroxidase-coupled secondary antibody (1:2000 dilution in TBS, 0.05% Tween 20; Amersham Pharmacia Biotech) for 1 h; and washed 25 min in TBS, 0.05% Tween 20; and bands were visualized using the ECL system (Amersham Pharmacia Biotech). Blots and autoradiograms were scanned using an Agfa Studioskop II with transparency module. Brightness and contrast were adjusted with the Agfa Fotolook software, and they were printed on a Linotronic 200P printer.

DNA Synthesis—DNA synthesis was determined as described previously (4) with minor modifications. Differentiated 3T3-L1 adipocytes were transduced with adenoviruses. Cells were incubated in serum-free DMEM with 1% bovine serum albumin with or without 500 nM insulin for 17 h. The medium was replaced by DMEM and 1% bovine serum albumin with or without 100 nM insulin. After an additional 1-h incubation, cells were washed two times with cold PBS and lysed in 0.1% SDS. Lysates were precipitated with trichloroacetic acid, filtered through glass fiber filters (Whatman, GF/C), and washed with 10% (v/v) trichloroacetic acid. After a final rinse with ethanol, filters were dried, and tritium incorporation into the filtered material was determined by liquid scintillation counting.

Statistical Analysis—Statistical analysis was performed using Student’s two-tailed t test for unpaired comparisons utilizing Statview software.

RESULTS
Expression of Fusion Constructs in COS-7 Cells—To test for expression of the inter-SH2-GLUT4 chimeras, the expression plasmids containing the fusion constructs iSH2-G4, G4-, iSH2-G4, and G4-, iSH2-G4, (Fig. 1A) were transiently transfected in COS-7 cells, and lysates were used for Western blotting using antisera against either a C-terminal peptide of GLUT4 or an N-terminal peptide (right). Immunoreactivity corresponding to iSH2-G4, G4-, iSH2-G4, is indicated by arrows on the right; molecular mass markers in kilodaltons are indicated at the left.

Targeting PI3K to GLUT4 Vesicles

![Fig. 1. Expression of iSH2-GLUT4 fusion proteins in COS-7 cells. A, the predicted topology of GLUT4 in the membrane bilayer and position of critical amino acids (AA) for the cloning strategy are shown at the top. Fusion constructs were made by ligating the C-terminal amino acids 406–509 of GLUT4 (G4), with or without the N-terminal amino acids 1–10 of GLUT4 (G4-), to the iSH2, as described under “Experimental Procedures.” B, cells were transfected with plasmids encoding iSH2-G4, G4-, iSH2-G4-, or empty plasmid (control) and were homogenized after 48 h. 25 μg of homogenate protein were analyzed for expression by Western blotting, using anti-GLUT4 antiserum against either a C-terminal GLUT4 peptide (left) or an N-terminal GLUT4 peptide (right). Immunoreactivity corresponding to iSH2-G4, G4-, iSH2-G4, is indicated by arrows on the right; molecular mass markers in kilodaltons are indicated at the left.](image-url)
peptide of GLUT4. As shown in Fig. 1B, the C-terminal-specific GLUT4 antiserum detects immunoreactive bands of the expected molecular mass for both iSH2-G4c (45 kDa) and G4n-iSH2-G4c, while the N-terminal specific antiserum detects only G4n-iSH2-G4c. This demonstrates that both fusion constructs express the expected chimeric proteins in these cells. The 53-kDa band detected by the C-terminal antiserum is nonspecific and is present in nontransfected control cells.

Expression and Localization of Fusion Constructs in 3T3-L1 Adipocytes—To express targeted constitutively active PI3K in 3T3-L1 adipocytes, recombinant, replication-deficient adenoviruses encoding iSH2, iSH2-G4c, or G4n-iSH2-G4c, and p110 were prepared as described under “Experimental Procedures.” 6

Differentiated 3T3-L1 adipocytes were transduced overnight with recombinant adenoviruses encoding iSH2-G4c or G4n-iSH2-G4c together with p110. When compared with GLUT4, a significantly higher fraction of the fusion proteins is present in the PM even in the basal state. Fig. 3 shows that in the absence of insulin and without co-expression of p110, both fusion constructs are located in both the PM and LDM fractions, the major fractions involved in the translocation of GLUT4. Negligible amounts of the fusion constructs were detected in cytosol (not shown).

To further define the membrane distribution of the fusion proteins, 3T3-L1 adipocytes transduced with iSH2-G4c or G4n-iSH2-G4c-encoding adenovirus were subjected to subcellular fractionation by differential centrifugation. Western blotting of these fractions using GLUT4 antiserum (C-terminal) is shown in Fig. 3. Fig. 3A shows that insulin treatment increases both cytosolic and membrane-associated PI3K activity, while untargeted iSH2/p110 coexpression increases both cytosolic and membrane-associated PI3K activity (Fig. 2). In contrast, both iSH2-G4c/p110 and G4n-iSH2-G4c/p110 coexpression increase PI3K activity almost exclusively in the membrane fraction. Thus, fusion of the C-terminal portion of G4n-iSH2-G4c in the PM and a decrease in the LDM in
parallel with GLUT4, although to a smaller extent. A similar insulin-induced translocation was also seen for the other fusion protein, iSH2-G4c, when expressed without p110 (not shown). Fig. 3C shows that coexpression with p110 did not alter the distribution of the fusion protein iSH2-G4c, in the basal state or after insulin-stimulation. Similar results were obtained with co-expression of G4n-iSH2-G4c and p110 (not shown). We previously showed (4) that the nontargeted iSH2/p110 heterodimer was present primarily in LDM and cytosol and that insulin stimulation did not alter its subcellular distribution.

Expression of Targeted PI3K Activity in 3T3-L1 Adipocytes Increases PI3K Activity in GLUT4 Vesicles—To determine whether the fusion proteins were located in the GLUT4-containing vesicles and whether they increased PI3K activity in these vesicles when coexpressed with p110, GLUT4 vesicles were immunoadsorbed from an LDM/cytosol fraction (Fig. 4, left panel). Because both the C and N termini of GLUT4 were used in the fusion constructs, antiserum recognizing these regions of GLUT4 could not be used for immunoadsorption studies, since these antisera would also adsorb chimeric proteins that were not in GLUT4 vesicles. Hence, we used an affinity-purified antibody against the insulin-responsive aminopeptidase vp165 to prepare GLUT4 vesicles. It has previously been demonstrated in adipocytes that vp165 colocalizes with GLUT4 and is present exclusively in GLUT4 vesicles (30). Fig. 4 shows the results of adsorption of vesicles from iSH2-G4c/p110- or G4n-iSH2-G4c/p110-overexpressing cells. Western blotting indicated that both fusion proteins and GLUT4 were adsorbed with the vp165 antibody, with minimal nonspecific adsorption with control rabbit IgG (Fig. 4, left panel). The right panel of Fig. 4 shows that adsorption with vp165 antibody removes nearly all of iSH2-G4c, G4n-iSH2-G4c, and GLUT4 from the LDM/cytosol fraction, so these proteins are barely detectable in the supernatant. In contrast, after immunoadsorption with nonspecific rabbit IgG (last lane), very little G4n-iSH2-G4c, or GLUT4 is removed.

Fig. 5A shows a dramatic enrichment in PI3K activity in GLUT4 vesicles in cells transfected with adenoviruses encoding iSH2-G4c, or G4n-iSH2-G4c, with p110, while GLUT4 vesicles from nontargeted iSH2/p110-expressing cells show very minimal enrichment in PI3K activity. All results are in the absence of insulin and are compared with control cells expressing β-galactosidase. Fig. 5A shows a representative thin layer chromatograph, and Fig. 5B shows quantitation of PI3K activity from three independent experiments. Expression of either nontargeted or targeted iSH2 with p110 resulted in a 5.0–6.7-fold increase in PI3K activity in the homogenate. However, nontargeted iSH2/p110 resulted in an ~5-fold increase in activity in the LDM/cytosol fraction with only a 2-fold increase in GLUT4 vesicles. In contrast, coexpression of iSH2-G4c, or G4n-iSH2-G4c, with p110 resulted in a ~2-fold increase in activity in LDM/cytosol but an 11–13-fold increase in the GLUT4 vesicles. Thus, a large proportion of the iSH2-G4c and G4n-iSH2-G4c, which was present in the LDM/cytosol was targeted to GLUT4 vesicles. The additional increase in PI3K activity in the homogenate from cells cotransduced with the fusion constructs and p110 can be largely accounted for by increased activity in the PM (data not shown).

We next tested the effects of insulin on total PI3K activity in GLUT4 vesicles of nontransduced adipocytes. A previous study showed an increase in IRS-1-associated PI3K activity in GLUT4 vesicles (18). Insulin stimulation (2 and 4 min) led to a small (1.7 ± 0.2-fold and 1.4 ± 0.1-fold, respectively; mean ± S.E., n = 3) and transient increase in PI3K activity in GLUT4 vesicles as shown in Fig. 5C. In addition, the experiments shown in Fig. 5C demonstrate the presence of phosphoinositide 4-kinase activity in GLUT4 vesicles consistent with the previous detection of phosphoinositide 4-kinase immunoreactivity (37). As expected, phosphoinositide 4-kinase activity, unlike PI3K activity, is not altered by insulin treatment.

Glucose Transport in 3T3-L1 Adipocytes Expressing Targeted PI3K Activity—The effect of iSH2/p110, iSH2-G4c/p110, or G4n-iSH2-G4c/p110 coexpression on glucose transport is shown in Fig. 6. iSH2/p110 coexpression leads to a 5–6-fold increase in basal glucose transport as previously reported (4), while either fusion construct coexpressed with p110 induces only an ~2-fold increase in basal glucose transport. Neither nontargeted or targeted iSH2 altered maximally insulin-stimulated glucose transport. In addition, coexpression of the fusion constructs with p110 did not induce a measurable translocation of GLUT4 to the PM in the absence of insulin (Fig. 3C), while we showed that iSH2/p110 coexpression increases GLUT4 immunoreactivity in the PM ~3-fold (4). Expression of GLUT4 and GLUT1, assessed by Western blotting of total membrane fractions, was not altered by coexpression of iSH2, iSH2-G4c, or G4n-iSH2-G4c, with p110 when compared with nontransduced control cells or cells transfected with β-galactosidase encoding control virus (not shown). These results indicate that targeting PI3K activity to GLUT4 vesicles is less efficient in stimulating glucose transport and GLUT4 translocation than untargeted PI3K activity.

DNA Synthesis—To determine whether the fusion constructs have biological activity in intact cells, we measured the effects on DNA synthesis, which we recently showed was markedly increased by expression of nontargeted constitutively active PI3K in 3T3-L1 adipocytes (4). Fig. 7 shows that in cells that were not transduced (no virus) or were transduced with β-galactosidase, insulin stimulates thymidine incorporation ~5-fold. Both the nontargeted iSH2 and the targeted iSH2-G4c, expressed with p110 result in an ~10-fold increase in thymidine incorporation. Insulin further stimulates thymidine incorporation ~2-fold above basal levels in cells expressing either targeted or untargeted PI3K. This indicates that in intact cells, as well as in vitro (Fig. 5), both the targeted and untargeted
Targeting PI3K to GLUT4 Vesicles

PI3K activity by $^{32}$P incorporation into phosphatidylinositol and separation of phospholipids by thin layer chromatography. Positions of the origin, phosphatidylinositol 3-phosphate (PI3P), and phosphatidylinositol 4-phosphate (PI4P) are indicated on the left of the autoradiogram. All results are representative of three independent experiments.

FIG. 5. Coexpression of iSH2-GLUT4 fusion proteins with p110 in 3T3-L1 adipocytes increases PI3K activity in GLUT4 vesicles. Differentiated 3T3-L1 adipocytes were transduced overnight with the indicated recombinant adenoviruses, and following an additional 24 h, which included 16 h of serum starvation, cells were homogenized, and GLUT4 vesicles were prepared as described under "Experimental Procedures." A, homogenate and LDM/cytosol protein representing 1% of the cells from one 10-cm diameter plate and immunoabsorbed material representing 25% of the cells from one plate were used for determination of PI3K activity by $^{32}$P incorporation into phosphatidylinositol and separation of phospholipids by thin layer chromatography. Positions of the origin, phosphatidylinositol 3-phosphate (PI3P), and phosphatidylinositol 4-phosphate (PI4P) are indicated on the left of the autoradiogram. B, quantitation of PI3K activity in three experiments. PI3K activity (mean ± S.E.) in homogenate, LDM/cytosol, and GLUT4 vesicles from cells coexpressing iSH2/p110 (light gray), iSH2-G4c/p110 (dark gray), and G4n-iSH2-G4c/p110 (black) is expressed as fold increase over basal values in control cells expressing β-galactosidase (β-galactosidase). Basal values for each fraction are set to 1. C, insulin-stimulated PI3K activity in GLUT4 vesicles. Following 16 h of serum starvation, differentiated 3T3-L1 adipocytes were or were not stimulated with 100 nM insulin for 2 or 4 min. Cells were homogenized, and GLUT4 vesicles and a LDM/cytosol fraction were prepared as described under "Experimental Procedures." LDM/cytosol protein representing 1% of the cells from one 10-cm diameter plate and immunoabsorbed material representing 25% of the cells from one plate were used for determination of PI3K activity by $^{32}$P incorporation into phosphatidylinositol and separation of phospholipids by thin layer chromatography. Positions of the origin, phosphatidylinositol 3-phosphate (PI3P), and phosphatidylinositol 4-phosphate (PI4P) are indicated on the left of the autoradiogram. All results are representative of three independent experiments.

FIG. 6. 2-Deoxyglucose uptake in differentiated 3T3-L1 adipocytes coexpressing iSH2-GLUT4 fusion proteins and p110. Cells were transduced with the indicated recombinant adenoviruses overnight. After an additional 24 h, which included 3 h of serum-free incubation, cells were (black bars) or were not (gray bars) stimulated with 100 nM insulin for 30 min. 2-Deoxyglucose uptake was determined over a 10-min period as described under "Experimental Procedures." Data are presented as mean ± S.E. and are representative of five independent experiments, each performed in triplicate. beta-gal, β-galactosidase.

FIG. 7. Stimulation of DNA synthesis in differentiated 3T3-L1 adipocytes coexpressing untargeted iSH2 or iSH2-GLUT4 fusion protein and p110. Cells were transduced with the indicated recombinant adenoviruses overnight. They were then incubated in DMEM with 10% fetal calf serum for 24–48 h, followed by 17 h of incubation with (black bars) or without (white bars) 500 nM insulin in serum-free media with 1% bovine serum albumin. Thymidine incorporation was determined over 1 h as described under "Experimental Procedures." Data are presented as mean ± S.E. and are representative of three independent experiments, each performed in triplicate. beta-gal, β-galactosidase.
PI3K activation in intact cells. Furthermore, the targeted construct has similar capability as the untargeted construct to stimulate at least one biological effect, i.e. DNA synthesis.

**DISCUSSION**

Stimulation of PI3K is necessary for insulin-stimulated GLUT4 translocation and glucose transport (1–3), and more recently we (4) and others (5–7) demonstrated that constitutive activation of PI3K is sufficient to at least partially stimulate these responses. In contrast, PDGF or IL4 stimulation of insulin-responsive cells is not sufficient to elicit GLUT4 translocation or glucose transport despite stimulating PI3K activity (8, 9). Possible explanations for these discrepancies are 1) different levels, 2) different location, or 3) different time course or duration (38) of PI3K activation in response to different growth factors; 4) coactivation of other pathways that inhibit glucose transport by growth factors other than insulin; and/or 5) coactivation by insulin but not by other growth factors of additional pathways that are also necessary for maximal stimulation of glucose transport.

The specificity of growth factor or cytokine action is largely a function of the ligand-receptor interaction. One potential mechanism for preserving specificity downstream of the receptor and hence for producing a distinct pattern of cellular responses to a specific ligand is compartmentalization of signaling pathways. Insulin-stimulated PI3K activity resides primarily in an intracellular membrane compartment (10–13), while other growth factors such as PDGF stimulate PI3K activity primarily in plasma membranes (11, 12). It seems likely that this specific localization of PI3K activity has important implications for the point of insulin stimulation that was studied (19).

In agreement with the recent finding by Heller-Harrison et al. (18), we also find that insulin increases PI3K activity in the GLUT4-containing vesicles, a subfraction of the LDM. While two other studies using sucrose gradients do not find colocalization of the majority of IRS-1 or p85 with GLUT4 in adipocyte LDM (10, 19), one of these studies finds some overlap (10), and both studies could have missed the association due to its transient nature (once PI3K is activated in the vesicle, the vesicle translocates to the PM) or due to technical aspects of the experiments causing dissociation of protein-membrane complexes. The stimulation of PI3K that we and others (18) see in association with GLUT4 vesicles is relatively small (Fig. 5C) and most likely does not represent the majority of insulin-stimulated PI3K activity in the cell. However, even if the GLUT4 vesicle is not the major site of insulin-stimulated PI3K activity, one might hypothesize that activation of PI3K in the GLUT4 vesicle could potentiate GLUT4 translocation. Our current study shows that the iSH2-GLUT4 fusion proteins coexpressed with p110 target PI3K activity to GLUT4 vesicles and increase total PI3K activity 11–13-fold in these vesicles with less than a 2-fold effect in the larger LDM/cytosol fraction. However, this results in less efficient GLUT4 translocation and stimulation of glucose transport than untargeted coexpression of iSH2 and p110, which increases PI3K activity only 2-fold in GLUT4 vesicles but ~5-fold in LDM/cytosol. In agreement with the previous conclusion of Kelly and Ruderman (10), this suggests that stimulation of PI3K in different or additional subfractions of the LDM is important for insulin’s effect on GLUT4 translocation. Our data are also consistent with the possibility recently raised by Clark et al. that the IRS-1 and PI3K that pellet with the LDM are not actually associated with membranes but instead with the cytoskeleton, at least at the time point of insulin stimulation that was studied (19).

Since the fusion proteins can also be found in PM and increase PI3K activity in this fraction, unlike the untargeted iSH2-p110 coexpression, these results further support the finding that PI3K associated with the plasma membrane is not sufficient to stimulate glucose transport. This is consistent with the hypothesis that the inability of PDGF to stimulate glucose transport is due, at least in part, to the lack of stimulation of PI3K in the LDM. It is also possible that PDGF coactivates other pathways that inhibit glucose transport, although no such pathways have been identified.

It might be argued that the magnitude of the increase in PI3K activity in GLUT4 vesicles as a result of the targeted fusion proteins is so high that it could nonspecifically block translocation. Similarly, the persistent rather than transient activation could block translocation. Two observations argue against these possibilities: 1) the insulin response is not impaired in cells coexpressing high levels of p110 and either of the fusion constructs (Figs. 3 and 6), and 2) using lower amounts of viruses and thereby decreasing expression levels of the fusion proteins further diminished the small stimulatory effect on glucose transport, rather than increasing it (not shown).

It is remarkable that the addition of the last two transmembrane regions and the C-terminal tail of GLUT4 is sufficient to target an otherwise predominantly cytosolic protein almost completely to membranes and at least partially to GLUT4 vesicles. The importance of the C-terminal tail, and specifically the double leucine motif, for GLUT4 sorting and intracellular retention has previously been described (21, 22, 24). In addition, motifs in central sections of GLUT4 (39) and the 19 N-terminal amino acids (23) are thought to be involved in targeting of the GLUT4 protein. The fact that the addition of the N-terminal tail did not alter the subcellular distribution of the fusion construct could be due to the fact that the tertiary structure of the N-terminal tail in the fusion protein does not allow “normal” interaction with other proteins or that additional regions of GLUT4 are necessary to form a functional motif. The presence of the targeted constructs in the PM is actually not surprising. Although the C- and N-terminal regions confer GLUT4-like sequestration to the chimeric proteins, the rates of internalization and endosomal trafficking may not be identical to GLUT4, since the chimeric proteins do not have all of the structural elements contained in GLUT4. Indeed, single amino acid substitutions in GLUT4 have been shown to alter recycling rates and result in increased GLUT4 in the PM (23).

The predominant localization in the PM was not due to increased PI3K activity associated with the fusion constructs, since both incubation with wortmannin (not shown) and expression of the fusion proteins without coexpression of p110 (and hence without catalytic activity; Fig. 3) did not alter the subcellular distribution. In parallel, expression of untargeted iSH2 without p110 did not increase PI3K activity in membranes or cytosol either (4). We also considered the possibility that relatively short term, high level increase in PI3K activity might initially saturate the sorting process without allowing time to achieve a new steady state distribution. Therefore, we expressed the fusion proteins for 10 days instead of the usual 1.5 days. This did not lead to a substantial redistribution of iSH2-GLUT4 fusion proteins from the PM to the LDM (not shown).

Despite the fact that high levels of PI3K activity can be measured in vitro (Fig. 5), one might be concerned that targeting could position the PI3K enzyme in a location where it no longer had access to its lipid substrates or could change its orientation in membranes so it would not be activated in intact cells. The marked stimulation of DNA synthesis in intact adipocytes (Fig. 7) indicates that both the targeted and untargeted PI3K constructs are biologically active. In summary, by fusing the C-terminal tail of GLUT4 to the iSH2 region of PI3K and
coexpression of p110, we successfully targeted constitutively active PI3K to GLUT4-containing vesicles. While targeted and untargeted constructs had similar effects to stimulate DNA synthesis, targeted activation of PI3K led to a much smaller increase in glucose transport compared with untargeted PI3K activation and no detectable GLUT4 translocation to the PM. These results argue against a critical role for localization of PI3K in the GLUT4 vesicle in triggering GLUT4 translocation. They suggest that the unique effect of insulin to stimulate PI3K in the GLUT4 vesicle in triggering GLUT4 translocation.

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