Membrane-targeted Phosphatidylinositol 3-Kinase Mimics Insulin Actions and Induces a State of Cellular Insulin Resistance*

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Phosphatidylinositol (PI) 3-kinase plays an important role in various insulin-stimulated biological responses including glucose transport, glycogen synthesis, and protein synthesis. However, the molecular link between PI 3-kinase and these biological responses is still unclear. We have investigated whether targeting of the catalytic p110 subunit of PI 3-kinase to cellular membranes is sufficient and necessary to induce PI 3-kinase dependent signaling responses, characteristic of insulin action. We overexpressed Myc-tagged, membrane-targeted p110 (p110CAAX), and wild-type p110 (p110WT) in 3T3-L1 adipocytes by adenovirus-mediated gene transfer. Overexpressed p110CAAX exhibited ~2-fold increase in basal kinase activity in p110 immunoprecipitates, that further increased to ~4-fold with insulin. Even at this submaximal PI 3-kinase activity, p110CAAX fully stimulated p70 S6 kinase, Akt, 2-deoxyglucose uptake, and Ras, whereas, p110WT had little or no effect on these downstream effects. Interestingly p110CAAX did not activate MAP kinase, despite its stimulation of p21ras. Surprisingly, p110CAAX did not increase basal glycogen synthesis activity, and inhibited insulin stimulated activity, indicative of cellular resistance to this action of insulin. p110CAAX also inhibited insulin stimulated, but not platelet-derived growth factor-stimulated mitogen-activated protein kinase phosphorylation, demonstrating that the p110CAAX induced inhibition of mitogen-activated protein kinase and insulin signaling is specific, and not due to some toxic or nonspecific effect on the cells. Moreover, p110CAAX stimulated IRS-1 Ser/Thr phosphorylation, and inhibited IRS-1 associated PI 3-kinase activity, without affecting insulin receptor tyrosine phosphorylation, suggesting that it may play an important role as a negative regulator for insulin signaling. In conclusion, our studies show that membrane-targeted PI 3-kinase can mimic a number of biologic effects normally induced by insulin. In addition, the persistent activation of PI 3-kinase induced by p110CAAX expression leads to desensitization of specific signaling pathways. Interestingly, the state of cellular insulin resistance is not global, in that some of insulin’s actions are inhibited, whereas others are intact.

One of the major physiological functions of insulin is to stimulate glucose transport into insulin-sensitive tissues by eliciting translocation of the major insulin responsive glucose transporter, GLUT4 from an intracellular compartment to the plasma membrane (1, 2). However, the signaling events that mediate insulin-stimulated glucose transport and GLUT4 translocation are poorly understood. Binding of insulin to its receptor results in receptor autophosphorylation and activation of the receptor tyrosine kinase, followed by tyrosine phosphorylation of several intermediate proteins, such as, insulin receptor substrates: IRS-1, 2, 3, 4, and the adaptor protein, Shc (3–5). Tyrosine-phosphorylated insulin receptor substrates and the insulin receptor itself, then bind to Src homology 2 (SH2) domain containing proteins, which further propagates downstream signals.

Phosphatidylinositol (PI) 3-kinase, a dual protein and lipid kinase is one such signaling molecule (6). It consists of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. Several isoforms of the regulatory subunit, p55α, p55PK, p85α, p85β, and two isoforms of the catalytic subunit, p110α and p110β, have been identified. The p85 subunit is composed of an NH2-terminal Src homology 3 (SH3) domain and two SH2 domains (7). The SH2 domains flank the region where p110 associates with p85. The SH2 domains interact with phosphotyrosine residues leading to activation of the p110 catalytic subunit. The p110 subunit phosphorylates phosphoinositides at the 3’-position of the inositol ring to generate phosphoinositides 3-P, 3,4-P2, and 3,4,5-P3 (7). PI 3-kinase also phosphorylates proteins on serine/threonine residues (8–10).

Ras is another signaling protein downstream of IRS-1 and Shc. After insulin stimulation, tyrosine phosphorylated Shc, and IRS-1, to a lesser extent, interact with another SH2 domain containing adaptor protein-Grb2, which is pre-associated with Sos, a guanine nucleotide exchange factor that promotes the formation of the active GTP-bound state of Ras (11). Stimulated Ras then activates a cascade of protein serine/threonine kinases, which include Raf, MEK, and the MAP kinases. Although PI 3-kinase and Ras appear to be on separate pathways branching from IRS-1, both have been implicated in pathways that mediate the mitogenic actions of insulin, whereas the metabolic effects of insulin are primarily activated by PI 3-kinase dependent steps.

Several lines of evidence indicate that activation of PI 3-kinase by insulin is required for GLUT4 translocation. For ex-
ample, the PI 3-kinase inhibitors, wortmannin and LY 294002 prevent GLUT4 translocation and stimulation of glucose transport in rat and 3T3-L1 adipocytes (12–14). Dominant-negative mutants of the 85-kDa subunit of PI 3-kinase can also inhibit GLUT4 translocation in response to insulin (15–17). However, several other observations suggest that, although necessary, PI-3-kinase activation is not sufficient to promote glucose transporter translocation. Indeed, growth factors such as platelet-derived growth factor (PDGF) can stimulate PI-3-kinase as efficiently as insulin, but have only a minor effect on GLUT4 translocation (18, 19). Similarly, interleukin 4, which induces tyrosine phosphorylation of IRS-1 and PI-3-kinase activation, does not stimulate GLUT4 translocation in L6 myoblasts (18). Furthermore, subcellular fractionation analyses indicate that insulin, unlike other growth factors, stimulates PI-3-kinase activity not only in the plasma membrane fraction but also in the low density microsomal compartment (20–22) and possibly even in GLUT4 containing subfractions of the low density microsomal of adipocytes (23, 24). Thus, it appears that insulin-mediated subcompartmentalization of PI-3-kinase may be unique and might be key to the specificity of the effect of insulin on glucose transport.

The aim of this study was to determine whether targeting of PI-3-kinase catalytic subunit to membrane structures is sufficient to trigger signaling events downstream of PI-3-kinase. This allows us to directly study PI-3-kinase-regulated cellular processes in the absence of insulin and to determine whether PI-3-kinase activation is sufficient to trigger signaling events specific for insulin. Furthermore, it avoids potential problems associated with the use of PI-3-kinase inhibitors in elucidating the actions of this enzyme. We, and, others have recently demonstrated that increased PI-3-kinase activity induced by expression of a constitutively active p110 subunit (p110α) can induce GLUT4 translocation (25), but it stimulates glucose transport only partially in the absence of insulin (26). In contrast, our membrane localized form of the p110 subunit of PI-3-kinase resulted in activation of downstream mitogenetic effects in COS-7 cells (27). Since gene transfer in 3T3-L1 adipocytes by conventional methods is inefficient, we utilized adenovirus-mediated, high efficiency gene transfer procedures (26, 28, 29), and created an adenoviral vector containing the p110α subunit of PI-3-kinase which could be targeted to the COOH terminus in order to target the p110 subunit to cellular membranes. Our studies showed that expression of the membrane-targeted p110 subunit of PI-3-kinase in 3T3-L1 adipocytes was sufficient to induce PI-3-kinase dependent downstream signaling events, including glucose transport.

**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine insulin was kindly provided by Lilly. Phospho-specific MAP kinase antibody and phospho-specific p70 S6 kinase antibodies were from Santa Cruz Biotechnology, Inc. Erk-1 antibody and PY20H were from Transduction. Dulbecco’s medium (DMEM) and fetal calf serum (FCS) were obtained from Life Technologies. All radioisotopes were obtained from NEN Life Sciences. Antibodies and PY20H were from Transduction. Dulbecco’s medium (DMEM) and fetal calf serum (FCS) were obtained from Life Technologies. All radioisotopes were obtained from NEN Life Sciences. Antibodies and PY20H were from Transduction.

**Cell Culture**—3T3-L1 cells were grown and maintained in DMEM high glucose medium containing 50 units of penicillin/ml, 50 μg of streptomycin/ml, and 10% FCS in a 5% CO2 environment.

**PI 3-Kinase Assay**—3T3-L1 adipocytes were infected with Ad5-p110α, Ad5-p110WT, or Ad5-CT at the indicated m.o.i.s for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 52 h. Serum-starved (16 h) cells were incubated in the absence (basal) or presence of insulin (100 ng/ml) for 10 min, washed once with ice-cold phosphate-buffered saline, lysed, and subjected to immunoprecipitation (300–500 μg of total protein) with antibodies to p110α-CT, c-Myc (2 μg), or IRS-1 (4 μg) overnight at 4 °C. Immune complexes were precipitated from the supernatant with protein G plus (Santa Cruz) or protein A and affinity-purified (34). The complexes were incubated with phosphatidylinositol (Avanti) and γ-[32P]ATP (3000 Ci/mmol) for 10 min at room temperature. Reactions were stopped with 20 μl of 8 N HCl and 160 ml of CHCl3:methanol (1:1) and centrifuged, and the lower organic phase was removed and applied to a silica gel thin-layer chromatography (TLC) plate (Merck) which had been coated with 1% potassium oxide. TLC plates were developed in CHCl3:CH3OH:

**Plasmid Construction**—pSG5-p110α, and pSG5-p110WT were obtained as described previously (30). The 9E10 epitope was inserted at the unique Sac II terminus of bovine p110α (DN, and pLC-CCK-CVL(S)). The 9E10 epitope was inserted at the unique Sac II terminus of bovine p110α (DN, and pLC-CCK-CVL(S)). The 9E10 epitope was inserted at the unique Sac II terminus of bovine p110α (DN, and pLC-CCK-CVL(S)).

**Preparation of Recombinant Adenovirus**—The recombinant adenovirus was expressed from the cDNA encoding the p110α or p110WT, and were isolated by homologous recombination with two plasmids, pACCMVpLpA (31) and pJM17 (32) as described previously (29). The recombinant plasmids, pAC-p110α or pAC-p110WT, and pJM17 were purified and co-transfected into 293 cells. Since 293 cells were originally derived from adenovirus transformation, the missing E1 gene function of pJM17 is provided in trans. The resulting recombinant viruses containing the p110α or p110WT are denoted as Ad5-p110α or Ad5-p110WT, respectively, and are defective (at least in cells lacking the E1 region of adenovirus), but fully infectious.

**Detection of Recombinant Ad5-p110α and Ad5-p110WT or Wild-type Virus in Cell Culture Medium by PCR Amplification of Viral DNA**—DNA templates for PCR were extracted from the supernatant of the culture medium of the 293 cells that were infected with each plaque isolates/viruses at a multiplicity of infection (m.o.i.) of 50 plaque forming units/ml. A multiplex PCR was performed on 1/10 dilution of virus DNA using E1A and E2B region-specific primers (33) and analyzed for the presence of recombinant or wild-type adenovirus. The presence of p110α and p110WT cDNA inserts in the recombinant virus was confirmed by PCR analysis of viral DNA with p110α cDNA specific primers. One clone of each of the recombinant viruses was amplified further in 293 cells.

**Cell Treatment**—3T3-L1 adipocytes were transduced at a m.o.i. of 1–40 plaque-forming units/cell for 16 h with stocks of either a control recombinant adenovirus (Ad5-CT) containing the cytomegalovirus promoter, pUC 18 polylinker, and a fragment of the SV40 genome, the recombinant adenoviruses Ad5-p110α or Ad5-p110WT. Transduced cells were incubated for 48 h at 37 °C in 16% CO2 and DME high glucose medium with 2% heat-inactivated serum, followed by incubation in the starvation media required for the assay. The efficiency of adenovirus-mediated gene transfer was approximately 90% as measured by immunocytochemistry. The survival of the differentiated 3T3-L1 adipocytes was unaffected by incubation of cells with the different adenovirus constructs since the total cell protein remained the same in infected or uninfected cells.

**Western Blotting**—3T3-L1 adipocytes uninfected or infected with Ad5-p110α or Ad5-p110WT, or Ad5-CT were starved for 16 h in DMEM medium containing 0.05% FCS. The cells were stimulated with 100 ng of insulin/ml for 5–30 min at 37 °C and lysed in a solubilizing buffer containing 20 mM Tris, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P40, 0.02% SDS, 0.01% phosphate-buffered saline, lysed, and subjected to immunoprecipitation (34–36). The cells were then centrifuged to remove insoluble materials. For Western blot analysis, whole cell lysates (20–80 μg of protein per lane) were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and resolved by SDS-PAGE. Gels were transferred to nitrocellulose by electroblotting in Towbin buffer containing 0.02 SDS and 20% methanol. For immunoblotting, membranes were blocked and probed with specified antibodies. Blots were then incubated with horseradish peroxidase-linked antibody for chemiluminescence detection, according to the manufacturer’s instructions (Pierce).
H$_2$O-NH$_2$OH (60:47:11:3.2), dried, and visualized, and quantitated on a Molecular Dynamics PhosphorImager.

2-Deoxyglucose Transport—The procedure for glucose transport was a modification of the methods described by Klip et al. (35). Differentiated 3T3-L1 adipocytes were infected with Ad5-p1100CAAX, Ad5-p110WT, or Ad5-CT at the indicated m.o.i.s for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 72 h. Serum and glucose-deprived cells were incubated in MEM in the absence (basal) or presence of 100 ng of insulin/ml for 1 h at 37 °C. Glucose uptake was determined in duplicate or triplicate at each point after the addition of 10 μl of substrate (2-[^3H]deoxyglucose or L-[^3H]glucose; 0.1 μCi, final concentration 0.01 mmol/liter) to provide a concentration at which cell membrane transport is rate-limiting. The value for t-glucose was subtracted to correct each sample for the contributions of diffusion and trapping.

Glycogen Synthase Activity—Glycogen synthase activity was determined as described previously (36). Differentiated 3T3-L1 adipocytes were infected with Ad5-p110CAAX, Ad5-p110WT, or Ad5-CT at 40 m.o.i. for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 72 h. The cells were serum and glucose-starved in DMEM no glucose, 2 mM pyruvate medium for 3 h, then stimulated with or without 200 ng of insulin/ml for 30 min in 5 mM glucose containing medium. Cells were washed with ice-cold phosphate-buffered saline three times, scraped in the buffer containing 50 mM Tris-HCl, 100 mM potassium fluoride, pH 7.4, and sonicated. After centrifugation, protein concentration was measured. 10 μg of protein was used to determine the ability to stimulate incorporation of [14C]UDP-glucose into glycogen in the presence and absence of glucose 6-phosphate.

Ras GTP/GDP Assay—Differentiated 3T3-L1 adipocytes were infected with Ad5-p110CAAX or Ad5-CT at 30 m.o.i. for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 48 h. Following 24 h serum starvation, the cells were stimulated with or without insulin (100 ng/ml) for 10 min, washed with phosphate-buffered saline, scraped, and frozen at −70 °C immediately. Frozen cell pellets were extracted in ice-cold RIPA buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, leupeptin, and pepstatin) by shaking for 5 min at 4 °C. The resulting cell extracts were centrifuged at 10,000 × g for 2 min. The supernatants were divided in half and either 3 μg of the anti-Ras antibody Y13-259 (Santa Cruz Biotechnology) or 3 μg of rat IgG (Cappel) were added. To both samples, goat anti-rat IgG and protein G-agarose were added as well as NaCl, SDS, and deoxycholate to final concentrations of 500 mM, 0.85%, and 0.5%, respectively. The samples were shaken gently for 1 h at 4 °C and then the immunoprecipitates were washed 4 times in RIPA buffer containing 500 mM NaCl, 0.05% SDS, and 0.5% deoxycholate and 2 times in 20 mM Tris-PO$_4$, pH 7.4. The washed immunoprecipitates were resuspended in 30 μl of 5 mM Tris-PO$_4$, pH 7.4, 2 mM dithiothreitol, 2 mM EDTA (TED buffer), heated to 100 °C for 3 min, cooled on ice, and centrifuged at 10,000 × g for 2 min. The immunoprecipitates were washed with an additional 15 μl of TED buffer which was combined with the first 30 μl of TED buffer and GFP and GDP were measured as described below.

GTP was converted to ATP using NAD kinase and ADP with the ATP measured in the luciferase/luciferin system according to the following reactions,

\[
\text{NAD kinase} \quad \text{GTP} + \text{ADP} \rightarrow \text{GDP} + \text{ATP}
\]

\[
\text{luciferase} \quad \text{ATP} + \text{luciferin} \rightarrow \text{oxyluciferin} + \text{AMP} + \text{PPI} + \text{light}
\]

**Reaction 1**

where PPI is pyrophosphate. This assay is sensitive to 1 fmol of GTP and was performed essentially as described previously (37). GDP was converted to GTP using pyruvate kinase and phosphoenolpyruvate with the GTP measured as described above.

\[
\text{Pyruvate kinase} \quad \text{GDP} + \text{phosphoenolpyruvate} \rightarrow \text{GTP} + \text{pyruvate}
\]

**Reaction 2**

Because the final product is again emitted light, this assay is also sensitive to 1 fmol. The reaction mixture was incubated for 30 min at 30 °C and contained in a final volume of 15 μl of 50 mM glycine, pH 7.8, 10 mM dithiothreitol, 8 mM MgSO$_4$, 50 μM phosphoenolpyruvate, 3 milliunits of pyruvate kinase and 5 μl of sample or GDP standard. It should be noted that this assay measures the sum of GTP + GDP; thus, the amount of GTP in the sample must be subtracted from the amount of GDP. DNA was measured by a standard fluorescence method using the fluorescent dye bisbenzimide and protein was measured by the Bradford method.

The amounts of GDP and GTP in the samples are determined from standard curves prepared with each set of samples and the data are expressed as femtomoles of GTP or GDP per microgram of DNA or milligram of protein in the cell lysate.

## RESULTS

Expression of Ad5-p110CAAX and Ad5-p110WT in 3T3-L1 Adipocytes

Western Blot of Myc-tagged Proteins—The differentiated 3T3-L1 adipocytes were infected with recombinant adenoviruses expressing the membrane-localized p110CAAX and the wild-type p110WT at 40 m.o.i. for 16 h and protein expression was examined 72 h later by Western blotting. Immunoblotting was performed against the Myc-tagged epitope present at the amino terminus of both the recombinant constructs. Specific bands appeared at −110 kDa corresponding to the p110CAAX and the p110WT proteins expressed in infected 3T3-L1 adipocytes (Fig. 1, lanes 2 and 3). The level of expression of both the proteins was similar.

PI 3-Kinase Activity—The membrane-localized p110CAAX and the wild-type p110WT overexpressing 3T3-L1 adipocytes were incubated with or without insulin for 10 min and the PI 3-kinase activity was measured in anti-Myc and anti-p110α immunoprecipitates. A representative experiment utilizing anti-p110α immunoprecipitates is shown in Fig. 2A and quantitation of data from seven separate experiments is shown in Fig. 2B, where the PI 3-kinase activity is expressed as percent of the basal activity (observed in unstimulated, Ad5-p110CAAX infected cells). Overexpression of the membrane-localized p110CAAX protein resulted in a 2-fold increase in p110α-associated PI 3-kinase activity when infected at 40 m.o.i. in the absence of insulin (Fig. 2B). Insulin stimulation of these cells further increased PI 3-kinase activity up to 4-fold (Fig. 2B), whereas, the cells infected at the same m.o.i. with control adenovirus elicited only 2.5-fold increase in PI 3-kinase activity upon insulin stimulation. In contrast, the wild-type p110WT overexpression induced only a modest, but a dose-dependent elevation of the p110 α-associated PI 3-kinase activity in the
absence of insulin. Upon insulin treatment, the level of p110α-associated PI 3-kinase activity increased further, up to 2.5-fold in the p110WT expressing cells, similar to that observed in uninfected or control infected cells. Preincubation with 1 mM wortmannin blocked the p110-associated PI 3-kinase activity in the membrane-localized p110CAAX overexpressing 3T3-L1 adipocytes.

Biological Effects of p110CAAX

Ser/Thr Phosphorylation of Akt in 3T3-L1 Adipocytes—Akt is a serine/threonine kinase downstream of PI 3-kinase which is activated by serine/threonine phosphorylation. Akt has been implicated as a mediator of several metabolic effects of insulin, including GLUT4 translocation, glucose uptake, and glycogen synthase activation. Therefore, we determined whether PI 3-kinase can activate Akt, using the gel mobility shift assay. Cell lysates from 3T3-L1 adipocytes infected with increasing m.o.i. of the membrane-localized p110CAAX and the wild-type p110WT expressing adenoviruses, were analyzed by SDS-PAGE followed by Western blotting with anti-Akt antibody (Fig. 3). The retarded gel mobility indicates serine/threonine phosphorylation and activation of Akt. Overexpression of the p110CAAX led to a significant increase in Akt activation, in a dose-dependent manner (Fig. 3, lanes 7 and 8). The extent of Akt activation by p110CAAX at 40 m.o.i. was comparable to that observed with insulin alone (Fig. 3, lanes 2 and 8). Further addition of insulin had a modest additive effect on Akt activation (lane 9). In contrast, p110WT overexpression did not activate Akt (lanes 3–5). Expression of an empty adenoviral vector, Ad5-CT, did not affect Akt activity either in the basal or insulin-stimulated state (data not shown). The activation of Akt with insulin or with p110CAAX was completely inhibitable by treatment with wortmannin (Fig. 3, lanes 6 and 10), demonstrating that PI 3-kinase is necessary for Akt activation.

p70 S6 Kinase Activation—It has been shown that p70 S6 kinase, another serine/threonine kinase is downstream of PI 3-kinase and Akt and that activation of PI 3-kinase and/or Akt is necessary for p70 S6 kinase activation. We, therefore, exam-
independent experiments.

Sulin treatment had a small additive effect on p70 S6 kinase

3T3-L1 adipocytes, and overexpression of p110 WT was unable

ing p110C

viral vector (Ad5-CT). Thus, glucose transport in cells express-

basal uptake measured in cells infected with the empty adeno-

Fig. 4, Effects of overexpression of p110CAAX and p110WT on p70 S6 kinase activation in 3T3-L1 adipocytes. Cells were uninfected (−, lanes 1–3) or infected with Ad5-p110WT (p110WT, lanes 4–8), or Ad5-p110CAAX (p110caax, lanes 9–13) at the indicated m.o.i. for 16 h. Serum-starved (16 h) cells were pretreated with 1 μM wortmannin (Wort., lanes 8 and 13) or 20 nM rapamycin (Rapa., lane 1) for 30 min, and incubated in the absence or presence of insulin (100 ng/ml) for 30 min (lanes 1, 3, 7, and 12). Total cell lysates (30 μg) were subjected to SDS-PAGE and immunoblotted with p70 S6 kinase antibody (upper panel) or phospho-specific p70 S6 kinase antibody (lower panel). The Western blot is representative of six independent experiments.

ined the ability of the membrane-localized p110CAAX to activate

p70 S6 kinase by using both a mobility shift assay and the

phospho-specific antibody that detects p70 S6 kinase only when it is phosphorylated at Thr14310/Ser14314. The effect of p110CAAX

overexpression on p70 S6 kinase activation is parallel to that observed for Akt activation (Fig. 4). p110CAAX overexpression led to an insulin-independent activation of p70 S6 kinase in a dose-dependent manner (Fig. 4, upper panel, lanes 9–11). Insulin treatment had a small additive effect on p70 S6 kinase stimulation (lane 12). p110WT overexpression showed a modest effect to stimulate p70 S6 kinase mobility, but the extent of activation was much less than that exhibited by the p110CAAX protein or by insulin (lanes 4–6). The inhibitors rapamycin or wortmannin prevented insulin or p110CAAX stimulation of p70 S6 kinase. The phospho-specific p70 S6 kinase blot showed a similar pattern of p70 S6 kinase activation as observed with the mobility shift assay (Fig. 4, lower panel).

Glucose Transport Stimulation—3T3-L1 adipocytes were infected with Ad5-p110CAAX, Ad5-p110WT, or Ad5-CT and 2-[3H]deoxyglucose uptake was measured 86 h later, after treating cells with or without 100 ng/ml insulin for 1 h. In uninfected cells, insulin stimulated glucose uptake by ~8-fold, and this was not affected by Ad5-CT infection (Fig. 5). In contrast, the basal 2-deoxyglucose uptake in cells overexpressing p110CAAX was elevated by ~4–8-fold, compared with the basal uptake measured in cells infected with the empty adenoviral vector (Ad5-CT). Thus, glucose transport in cells expressing p110CAAX was almost the same as that observed after insulin treatment alone (Fig. 5). In contrast, 2-deoxyglucose uptake in 3T3-L1 adipocytes infected with p110WT was comparable with the uninfected cells, or with cells infected with the empty adenoviral vector, and was further stimulated up to 8-fold by insulin treatment. Thus, membrane-targeted p110CAAX mimics insulin-induced glucose transport activity in 3T3-L1 adipocytes, and overexpression of p110WT was unable to stimulate glucose uptake in the absence of insulin. Pretreatment with wortmannin inhibited p110CAAX and insulin-induced glucose transport. In addition, p110CAAX and p110WT overexpression did not have any effect on the expression levels of Glut 4, compared with the CT infected cells (data not shown).

p21CAAX Induces Cellular Insulin Resistance

p110CAAX Stimulates Ser/Thr Phosphorylation of IRS-1 and Inhibits Its Function—It has been reported that PI 3-kinase phosphorylates serine/threonine residues of IRS-1 (9); we, therefore, examined the effect of p110CAAX on IRS-1 by the gel mobility shift assay. p110CAAX expression caused a mobility shift of IRS-1 in the absence of insulin without tyrosine phosphorylation, indicating phosphorylation of Ser/Thr residues (Fig. 7A). In addition, p110CAAX expression inhibited insulin-stimulated IRS-1 Tyr phosphorylation, without affecting insulin receptor tyrosine phosphorylation (Fig. 7B).

To investigate whether this serine/threonine phosphorylation altered IRS-1 function, we measured the PI 3-kinase activity associated with IRS-1. As shown in Fig. 7C, expression of p110CAAX resulted in ~40% inhibition of insulin-stimulated PI 3-kinase activity in IRS-1 antibody immunoprecipitates. This is in contrast to the ~45–55% increase in p110CAAX induced PI 3-kinase activity observed in p110α antibody immunoprecipitates (Fig. 7C). The p110CAAX-induced inhibition of insulin-stimulated IRS-1 associated PI 3-kinase activity was also observed in the cell lysates immunodepleted of the c-Myc immune complexes (Fig. 7C).

Glycogen Synthase Activity—Insulin led to an about 2.5-fold increase in glycogen synthase activity measured in control 3T3-L1 adipocytes with the empty adenoviral vector, Ad5-CT (Fig. 8). In cells infected with p110CAAX, the basal level of glycogen synthesis was decreased by ~30% compared with control cells, while insulin-stimulated glycogen synthase activity was completely inhibited. In cells expressing p110WT, basal glycogen synthase activity was unchanged and insulin led to an about 1.5-fold stimulation; this degree of stimulation was less.
Role of PI 3-Kinase in Insulin Signaling

FIG. 5. 2-Deoxyglucose uptake in 3T3-L1 adipocytes infected with increasing m.o.i. of adenoviruses expressing p110CAAX and p110WT. Differentiated 3T3-L1 adipocytes were infected with Ad5-CT (ctrl), Ad5-p110WT (p110WT), or Ad5-p110CAAX (p110caax) at the indicated m.o.i. for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 72 h. Serum and glucose-deprived cells were pretreated with 1 μM wortmannin (Wort.) for 30 min in α-DMEM, and incubated in the absence or presence of 100 ng of insulin/ml for 1 h at 37 °C. Cells were then washed with glucose-free medium and 2-deoxyglucose uptake was measured. Each measurement was performed in duplicate or triplicate. Data are the mean ± S.E. of 10 different observations and each value was corrected for protein concentration.

FIG. 6. Membrane-targeted p110CAAX elevates insulin-independent levels of GTP-bound Ras in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were infected with Ad5-CT (ctrl) or Ad5-p110CAAX (p110caax) at 30 m.o.i. in medium containing heat-inactivated serum (2%) for 16 h. Following infection, cells were serum starved (16 h), incubated in the absence or presence of insulin (100 ng/ml) for 10 min, lysed, and immunoprecipitated with the anti-Ras antibody, Y13-259. Then, GTP-Ras and GDP-Ras were measured as described under “Experimental Procedures.” Results are expressed as mean ± S.E. of percentage of GTP-bound Ras (% GTP) from five independent experiments. Percent GTP was determined as GTP-Ras/(GTP-Ras + GDP-Ras) × 100.

MAP Kinase Phosphorylation—We investigated whether the Ras activation induced by p110CAAX expression was accompanied by activation of the downstream effectors Erk1 and 2. This was accomplished in infected cells and Ad5- p110CAAX-infected cells by assessing MAP kinase activation using a phospho-specific MAP kinase antibody (Fig. 9, upper panel). Insulin increased MAP kinase activation in uninfected and Ad5-CT-infected cells by ~15-fold. Expression of the membrane-targeted p110CAAX had no effect on the basal MAP kinase phosphorylation state, and almost completely inhibited insulin-induced phosphorylation and activation of both p44 and p42 MAP kinase (Fig. 9, lane 5). In contrast, PDGF stimulated MAP kinase phosphorylation was unchanged by p110CAAX expression (Fig. 9, lane 6). Expression of MAP kinase, as assessed by Western blotting with a polyclonal anti-Erk-1 antibody that recognizes both nonphosphorylated and phosphorylated forms, demonstrated that the protein levels were not altered by infection with either Ad5-CT or Ad5-p110CAAX (Fig. 9, lower panel).

DISCUSSION

In this study, we have used adenoviral-mediated gene transfer to assess whether targeting of the catalytic p110 subunit of PI 3-kinase to cellular membranes by incorporating a CAAX box at the COOH terminus (p110CAAX) is sufficient to induce PI 3-kinase dependent signaling responses, characteristic of insulin action, in 3T3-L1 adipocytes. We show that when appropriately targeted, even modest levels of PI 3-kinase are sufficient to trigger full activation of the downstream serine/threonine kinases, Akt and p70 S6 kinase, and also causes stimulation of glucose transport equal to the effect of insulin. Surprisingly, insulin-mediated glycogen synthase activity was completely blocked in cells expressing p110CAAX. Furthermore, p110CAAX stimulated serine/threonine phosphorylation of IRS-1, and inhibited IRS-1 associated PI 3-kinase activity. Another major finding is that the membrane-localized PI 3-kinase activity was sufficient to mimic insulin-induced formation of GTP-bound p21ras. Last, we found that expression of p110CAAX led to inhibition of insulin-mediated MAP kinase activation, whereas PDGF-mediated MAP kinase activation was unaffected. These results lead to several predictions and conclusions.

p110CAAX Mimics Insulin Actions—We demonstrate that membrane-targeted p110 (p110CAAX) promotes insulin-independent PI 3-kinase activity and is sufficient to maximally stimulate glucose uptake, in a wortmannin-sensitive manner. The level of 2-deoxyglucose uptake achieved in response to p110CAAX expression is comparable to that seen in insulin-stimulated, control adipocytes, whereas, non-targeted wild-type p110α (p110WT) had only a slight effect on 2-deoxyglucose uptake. Since, the p110 subunit of PI 3-kinase contains a COOH-terminal membrane targeting farnesylation sequence, it seems likely that the overexpressed p110CAAX protein results in increased PI 3-kinase activity predominantly in membrane fractions, similar to insulin stimulation of endogenous PI 3-kinase. This implies that glucose uptake is not merely a function of the amount of PI 3-kinase present, but that its appropriate membrane localization is critical as well. This conclusion is quite consistent with other kinds of studies in the literature. For example, PDGF, as well as other growth factors, can stimulate PI 3-kinase in 3T3-L1 adipocytes, equally well as insulin, but only insulin leads to glucose transport stimulation (18, 19). These findings suggested that insulin induced subcompartmentalization of PI 3-kinase is necessary for metabolic signal-
ing. Consistent with this, Frevert and Khan (26) showed that co-expression of both the catalytic p110α subunit and the inter-SH2 region of the p85 regulatory subunit of PI 3-kinase in 3T3-L1 adipocytes led to a much higher level of PI 3-kinase activity than seen with insulin stimulation alone, but it had only a partial effect to stimulate glucose transport without insulin. In addition, when PI 3-kinase was activated by thiophosphorylated peptides, corresponding to the phosphotyrosine binding motif of the p85 subunit of PI 3-kinase, only a minor effect on Glut 4 translocation was observed (40). Tanti et al. (41) co-transfected rat adipose cells by electroporation with epitope-tagged Glut 4 and with either a constitutively active (p110*) or a kinase inactive form of p110α (41). Co-transfection with the active version of p110* resulted in stimulation of epitope-tagged Glut 4 translocation, similar to insulin, and these workers also found that the p110α was localized to the same intracellular compartment as the endogenous PI 3-kinase. Taken together with our current results, these studies support the conclusion that active PI 3-kinase is sufficient to stimulate glucose transport activity, only if it is targeted to the proper subcellular membranous compartment.

We further examined other targets of insulin action which are thought to be downstream of PI 3-kinase. Akt is a serine/threonine kinase that is activated by insulin. It is activated by a dual mechanism involving the binding of PI-(3,4)-P2 to its PH domain, as well as by serine/threonine phosphorylation by one or more Akt kinases, which may, themselves, be stimulated by the lipid products of PI 3-kinase (42). Several lines of evidence suggest that Akt functions downstream of PI 3-kinase, e.g. insulin-stimulated Akt kinase activity is inhibitable by wortmannin, a PI 3-kinase specific inhibitor, and PDGF receptor

![Figure 7](Image 347x353 to 515x450)

**FIG. 7.** Membrane-targeted p110α (AAAX) stimulates Ser/Thr Phosphorylation of IRS-1 and inhibits IRS-1 associated PI 3-kinase activity. Differentiated 3T3-L1 adipocytes were infected with Ad5-CT (ctrl) or Ad5-p110α (AAAX) (p110caax) at 40 m.o.i. in medium containing heat-inactivated serum (2%) for 16 h. Following infection, cells were serum starved (16 h), incubated in the absence or presence of insulin (100 ng/ml) for 5 (A and B) or 10 (C) min. Total cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotted with IRS-1 antibody (A) or PY20H (B). The Western blot is representative of three independent experiments. The cell lysates were divided into three, and subjected to immunoprecipitation with antibodies to p110α, IRS-1, or Myc (C). After Myc antibody immunoprecipitation, the supernatants were collected, and immunoprecipitated with IRS-1 antibody. The washed immunoprecipitates were assayed for PI 3-kinase activity with PI as substrate, and the labeled PI-3 phosphate product (PI-3P) was resolved by thin-layer chromatography and visualized by autoradiography. C shows mean ± S.E. of three experiments and the data is expressed as percentage of the maximal activity (100%) observed in insulin-stimulated, Ad5-CT-infected cells. □, Ad5-ctrl; ■, Ad5-CAAX.

![Figure 8](Image 64x323 to 282x729)

**FIG. 8.** Membrane-targeted p110α (AAAX) prevents insulin-induced glycogen synthase activity in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were infected with Ad5-CT (ctrl), Ad5-p110α (p110WT), or Ad5-p110α (AAAX) (p110caax) at 40 m.o.i. for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 72 h. The cells were serum and glucose starved in DMEM, 0.1% BSA, 2 mM sodium pyruvate for 3 h, then stimulated with or without 200 ng of insulin/ml for 30 min in 5 mM glucose containing medium. Following which, the cells were scraped, sonicated, and centrifuged. The ability of the supernatant to stimulate incorporation of UDP-glucose into glycogen was determined in the presence and absence of glucose 6-phosphate. Results are expressed as mean ± S.E. of percentage of glycogen synthase index (%) GSI from three independent experiments, and each observation was performed in duplicate. Percent GSI was determined as: (activity without glucose 6-phosphate/activity with G-6-P) × 100.

![Figure 9](Image 50x158)

**FIG. 9.** Membrane-targeted p110α (AAAX) prevents insulin-induced MAP kinase phosphorylation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were uninfected (−) or infected with Ad5-CT (ctrl) or Ad5-p110α (AAAX) (p110caax) at 10 m.o.i. in medium containing heat-inactivated serum (2%) for 16 h. Following infection, cells were serum starved (16 h), incubated in the absence or presence of insulin (100 ng/ml, I) or PDGF (20 ng/ml, P) for 10 min, lysed, subjected to SDS-PAGE, and immunoblotted with phospho-specific MAP kinase antibody (upper panel). The membrane was stripped and reblotted with anti-Erk-1 antibody (lower panel). The Western blots are representative of five independent experiments.

![Image 14312](Image 14312)
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mutants that fail to activate PI 3-kinase, also fail to activate Akt. Overexpression of a constitutively active Akt in 3T3-L1 adipocytes results in increased glucose uptake and Glut 4 translocation in the absence of insulin (43). Consistent with these findings, our data show that Akt activation is dependent on PI 3-kinase activity, and that insulin and p110CAAXinduced Akt activation is inhibitory by wortmannin, indicating that Akt activation is dependent on PI 3-kinase enzymatic function.

We also determined whether PI 3-kinase-mediated Akt activation would lead to p70 S6 kinase stimulation, since it has been shown that p70 S6 kinase is stimulated by constitutively active Akt (8) and blocked by inhibitors of PI 3-kinase (12, 44). Indeed, we found that overexpression of the membrane-targeted p110CAAX led to activation of p70 S6 kinase, which was completely inhibitory by wortmannin (Fig. 4). Our results are supported by the study of Weng et al. (44), which showed that transfection of a constitutively active form of PI 3-kinase (p110*) into 293 cells resulted in a 20–30-fold increase in cellular PI 3-kinase activity, that resulted in activation of p70 S6 kinase by phosphorylation at Thr-252. Wortmannin resulted in selective dephosphorylation at Thr-252 concomitant with inhibition of p70 S6 kinase activity. Furthermore, Klippeletal. (27) reported that in COS-7 cells, expression of membrane-localized p110 is sufficient to trigger downstream responses, characteristic of insulin action, including stimulation of Akt and p70 S6 kinase. Their study further adds that these responses can also be triggered by expression of p110*, that is cytosolic, but exhibits a high specific activity. However, our data are consistent with the idea that PI 3-kinase downstream of Ras is upstream of PI 3-kinase (30), which is sufficient to trigger downstream responses characteristic of insulin action, including stimulation of Akt, p70 S6 kinase, and glucose transport.

The role of PI 3-kinase in Ras-mediated signaling is unclear. An association between p21ras and PI 3-kinase was first demonstrated by co-immunoprecipitation in insulin and insulin-like growth factor-1 stimulated, Ras-transformed epithelial cells by Sjølander et al. (45). Subsequently, Ras was shown to bind in vitro to the p110 subunit of PI 3-kinase by Rodriguez-Viciana et al. (46). However, the relative position of PI 3-kinase with respect to Ras is confusing. Conflicting data exists suggesting that PI 3-kinase could be upstream, downstream, or independent of Ras. These alternate results are perhaps related to cell-type differences. Rodriguez-Viciana et al. (30) reported that a point mutation of the p110 subunit of PI 3-kinase at the Ras-GTP binding site elevated PI 3-kinase activity in COS cells, and the interaction of Ras-GTP, but not Ras-GDP, with PI 3-kinase led to an increase in its enzymatic activity (30). These data suggest that Ras is upstream of PI 3-kinase. However, our data are consistent with the idea that PI 3-kinase is upstream and can activate Ras (Fig. 6). We find that membrane-targeted activated PI 3-kinase activates p21ras, resulting in increased formation of p21ras-GTP, equal to the effect of insulin. This interpretation is in agreement with earlier data from our own laboratory, in which we reported that microinjection of dominant-negative PI 3-kinase, or PI 3-kinase inhibitory antibodies, into rat fibroblasts inhibited insulin-induced fos induction, which was rescued by activated (T-24) Ras (47). Similarly, studies by Hu et al. (48) suggest that Ras is downstream of PI 3-kinase because transfection of constitutively active PI 3-kinase resulted in fos induction, which was blocked by both dominant-negative Ras and Raf. They also found elevated levels of GTP-bound Ras in cells transfected with constitutively active PI 3-kinase.

Cellular Insulin Resistance Induced by p110CAAX—Interestingly, we found that p110CAAX did not mimic all of insulin’s actions, and, in some cases led to a decrease in insulin signaling indicating a partial, and selective insulin resistant state. For example, we found that p110CAAX did not mimic the effect of insulin to stimulate glycogen synthesis. Not only did p110CAAX expression fail to enhance basal glycogen synthase activity, but it completely inhibited the ability of insulin to stimulate glycogen synthesis. Activation of glycogen synthase by insulin involves a coordinated response, including phosphorylation induced inactivation of glycogen synthase kinase 3 (GSK3) and activation of protein phosphatase 1, by phosphorylation of its G subunit (pp1G) (49). It has been suggested that GSK3 is a downstream target of Akt, which, in turn, is dependent on PI 3-kinase activity. Constitutively active Akt inhibits insulin’s ability to stimulate glycogen synthesis in 3T3-L1 adipocytes (43, 50, 51), and our data also show that activation of Akt by the membrane-localized p110CAAX is not sufficient to cause glycogen synthase activation in 3T3-L1 adipocytes. In theory, activated PI 3-kinase and Akt should inactivate GSK3 by phosphorylation leading to stimulation of glycogen synthase activity, whereas we, and others, show that p110CAAX or constitutively active Akt inhibits insulin effects on this enzyme (43, 50, 51). However, it has been shown recently that GSK3 expression is either very low, or absent in 3T3-L1 adipocytes (52–54). Therefore, a role for GSK3 in our results is problematic. Perhaps the low (or absent) expression of GSK3 explains why p110CAAX does not stimulate glycogen synthesis by itself. An alternate pathway for glycogen synthase activation involves pp1, which has been suggested to be downstream of the IRS-1/Shc-MAP kinase pathway by some investigators (55), but a number of reports have indicated that this is not the case (56). In addition, earlier results show that the MEK inhibitor PD98059 does not lead to a decrease in insulin stimulation of glycogen synthesis (57). Thus, a role for MAPK in the regulation of glycogen synthesis seems unlikely. Another possibility is that IRS-1 directly or through its interacting proteins, but independent of PI 3-kinase, might be involved in the inhibition of glycogen synthase activity. Indeed, we find that membrane-targeted p110CAAX serine/threonine phosphatylates IRS-1, which is inhibitory by wortmannin. This in turn prevents IRS-1 tyrosine phosphorylation and downstream signaling (Fig. 7).

Although the precise mechanisms underlying the p110CAAX induced resistance are unknown, the current results provide some interesting insights. First, despite the fact that p110CAAX stimulated Ras activation, it had no effect to stimulate MAP kinase phosphorylation, indicating a blockade of MAP kinase activation at a site downstream of Ras. Furthermore, in p110CAAX expressing cells, insulin had no effect to stimulate MAP kinase phosphorylation, compared with a robust stimulation in control cells. Since insulin is thought to stimulate MAP kinase activation by activation of Ras (11), these findings also point to a post-Ras blockade of the MAP kinase pathway. On the other hand, p110CAAX expression did not inhibit PDGF-stimulated MAP kinase phosphorylation, and this is consistent with the interpretation that PDGF can lead to MAP kinase activation through a Ras-dependent as well as a non-Ras dependent pathway (58), and we would propose that expression of p110CAAX inhibits only the Ras-dependent input into MAP kinase. These findings also demonstrate that the p110CAAX induced inhibition of MAP kinase and insulin signaling is specific, and not due to some toxic or nonspecific effect on the cells.

Taken together, our results are consistent with the view that p110CAAX expression inhibits the actions of insulin at a step distal to Ras activation, leading to inhibition of MAP kinase, and, possibly, glycogen synthase activation. Importantly, the
cellular insulin resistance induced by p110CAAX in these cells is not global. Thus, p110CAAX expression stimulated AKT as well as p70 S6 kinase phosphorylation, and insulin had a further effect when added to p110CAAX expressing cells. This would argue that this model of cellular insulin resistance is rather unique, in that some of the insulin signaling pathways are inhibited, whereas, others are intact. The fact that persistent activation of PI 3-kinase leads to desensitization of subsequent downstream events is reminiscent of the fact that hyperinsulinemia (either in vitro or in vivo) will also lead to a state of cellular insulin resistance. However, hyperinsulinemia-induced insulin resistance affects all of insulin's actions, whereas, persistent PI 3-kinase activation selectively inhibits specific insulin signaling. Since insulin's biologic effects are pleiotropic with engagement of multiple divergent signaling pathways, further study of these cells may enhance our understanding of which signaling pathways connect to which biologic effects.

In summary, our studies show that PI 3-kinase activity can mimic a number of biologic effects normally induced by insulin, but that membrane targeting of this enzyme is necessary for activation of these events. In addition, the persistent activation induced by p110CAAX expression leads to desensitization of specific signaling pathways. Interestingly, the state of cellular insulin resistance is not global, in that some of insulin's actions are inhibited, whereas others are intact.

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