Inhibition of Phosphatidylinositol 3-Kinase Activity by Adenovirus-mediated Gene Transfer and Its Effect on Insulin Action*

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Phosphatidylinositol 3-kinase (PI 3-K) is implicated in cellular events including glucose transport, glycogen synthesis, and protein synthesis. It is activated in insulin-stimulated cells by binding of the Src homology 2 (SH2) domains in its 85-kDa regulatory subunit to insulin receptor substrate-1 (IRS-1), and, others. We have previously shown that IRS-1-associated PI 3-kinase activity is not essential for insulin-stimulated glucose transport in 3T3-L1 adipocytes, and that alternate pathways exist in these cells. We now show that adenovirus-mediated overexpression of the p85N-SH2 domain in these cells behaves in a dominant-negative manner, interfering with complex formation between endogenous PI 3-K and its SH2 binding targets. This not only inhibited insulin-stimulated IRS-1-associated PI 3-kinase activity, but also completely blocked anti-phosphotyrosine-associated PI 3-kinase activity, which would include the non-IRS-1-associated activity. This resulted in inhibition of insulin-stimulated glucose transport, glycogen synthase activity and DNA synthesis. Further, Ser/Thr phosphorylation of downstream molecules Akt and p70 S6 kinase was inhibited. However, co-expression of a membrane-targeted p101CAAX with the p85N-SH2 protein rescued glucose transport, supporting our argument that the p85N-SH2 protein specifically blocks PI 3-kinase activity, and, that the signaling pathways downstream of PI 3-kinase are intact. Unexpectedly, GTP-bound Ras was elevated in the basal state. Since p85 is known to interact with GTPase-activating protein in 3T3-L1 adipocytes, the overexpressed p85N-SH2 peptide could titrate out cellular GTPase-activating protein by direct association, such that it is unavailable to hydrolyze GTP-bound Ras. However, insulin-induced mitogen-activated protein kinase phosphorylation was inhibited. Thus, PI 3-kinase may be required for this action at a step independent of and downstream of Ras. We conclude that, in 3T3-L1 adipocytes, non-IRS-1-associated PI 3-kinase activity is crucial for insulin’s metabolic signaling, and that overexpressed p85N-SH2 protein inhibits a variety of insulin’s ultimate biological effects.

Insulin binding to its cell surface receptors initiates diverse metabolic and mitogenic signals by activation of a complex signaling cascade of protein tyrosine and serine/threonine kinases, as well as lipid kinases (1, 2). Phosphatidylinositol (PI)3-kinase (PI 3-kinase), a dual protein and lipid kinase is a heterodimeric enzyme composed of a 110-kDa catalytic subunit (p110) associated with an 85-kDa regulatory subunit (p85). Two isoforms of the catalytic subunit (p110α and p110β) and several isoforms of the regulatory subunit (p55α, p55PIK, p85α, and p85β) have been cloned so far. The regulatory subunit contains several well known functional domains: one Src homology 3 (SH3) domain, homology to the breakpoint cluster region (ber) gene, two proline-rich motifs, and two Src homology region 2 (SH2) domains (3). The p55α isoform is insulin-responsive and is predominantly expressed in 3T3-L1 adipocytes (4).

The insulin receptor is a tyrosine kinase, which, when activated by insulin, phosphorylates cellular substrates such as IRS-1, IRS-2, IRS-3, IRS-4 and the protein Shc, etc. (5–8). IRS-1 is the most well characterized among members of the IRS family. Following insulin stimulation, the phosphorylated YXXM motifs in IRS-1 binds to the SH2 domains of p85 stimulating the lipid kinase activity of the p110 subunit. Through the same SH2 mechanism, PI 3-kinase can associate with other proteins such as IRS-2–4, and the insulin receptor itself (9, 10). PI 3-kinase phosphorylates phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate, and phosphatidylinositol-4,5-biphosphate on the D-3 position of the inositol ring producing phosphatidylinositol 3-monophosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate, respectively. Insulin induces the production of D3-phosphorylated phosphoinositides through stimulation of PI 3-kinase activity in various tissues and cultured cells.

Insulin is a pleiotropic hormone that initiates a variety of biologic effects through a complex set of signaling cascades. As a general view, however, two main branches of the insulin signaling pathway can be activated in most cell types: one is controlled predominantly by PI 3-kinase (1, 11) and the other by the small GTP-binding protein p21ras (1). The major route by

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which insulin stimulates PI 3-kinase activity is through tyrosine phosphorylation of IRS-1, as described above. Insulin stimulates Ras activity through a dual mechanism involving Shc, and to a lesser extent, IRS-1, which subsequently binds to the Grb2/SOS complex, activating p21ras (12). Stimulated Ras then activates a cascade of protein serine/threonine kinases which includes Raf, MEK, and the mitogen-activated protein (MAP) kinases, ERK1 and ERK2 (reviewed in Refs. 1, 5, and 6). Activation of both of these pathways appears to be necessary for the mitogenic effects of insulin (12, 14, 15), whereas the metabolic effects of insulin are primarily activated by PI3-kinase-dependent steps (16, 17). Thus, it has been shown that activated PI3-kinase is both necessary and sufficient for insulin-stimulated GLUT4 translocation and glucose uptake (18–23), and downstream targets of PI3-kinase such as AKT and p70 S6 kinase may play important roles in the ultimate metabolic actions of this hormone (24, 25).

By no means are the PI 3-kinase and Ras/MAP kinase pathways completely separate. For example, Yamauchi et al. (26) have demonstrated that PI 3-kinase may lie upstream of Ras and in mediating mitogenic effects of insulin. On the other hand, experiments using wortmannin to inhibit PI 3-kinase activity have shown that the effect of insulin on p21ras-GTP loading in CHO cells is independent of PI 3-kinase (27). However, reciprocal relationships between PI 3-kinase and p21ras have also been demonstrated (28). Thus, one report has shown that activated Ras can stimulate PI 3-kinase activity, whereas other investigators have shown that the biologic consequences of constitutively activated PI 3-kinase are Ras-dependent (29). Thus, although the exact relationship between PI 3-kinase and p21ras is unclear, abundant data exist to indicate that they are interconnected in some way (30).

In the current studies, we have extensively evaluated the role of PI 3-kinase activity in a diverse array of insulin actions. Our approach was based upon the notion that cellular overexpression of the N-SH2 domain of the p85 subunit would disrupt complex formation between endogenous PI 3-kinase and its SH2 domain binding targets, such as IRS-1. This, in turn, would prevent insulin activation of PI 3-kinase enzymatic activity, allowing us to elucidate the biologic actions of this enzyme in insulin target cells such as 3T3-L1 adipocytes. Since gene transfer into 3T3-L1 adipocytes can be problematic, we elected to use recombinant adenovirus to achieve these goals. We (31), and others (32), have recently demonstrated that adenovirus mediated gene delivery allows high efficiency gene transduction and protein expression in terminally differentiated 3T3-L1 adipocytes. Thus, we prepared a recombinant adenovirus encoding the N-SH2 domain of the p85 α subunit of PI 3-kinase in order to overexpress this protein domain within 3T3-L1 adipocytes. Using this approach, we have investigated the acute effects of interrupting p85 association with insulin-stimulated target proteins on the ultimate biologic effects of insulin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine insulin was kindly provided by Lilly. Mouse monoclonal anti-phosphotyrosine (PY-20), mouse monoclonal anti-Erk1, and the anti-Shc-SH2 antibodies were purchased from Transduction Laboratories. Anti-IRS-1 and anti-human S6 kinase (p70S6K) were from Upstate Biotechnology Inc. (UBI). Phosphospecific MAPK and p70S6K antibodies were from New England Biolabs, Inc. The goat polyclonal AKT (C-20) antibody of human origin, anti-Ras antibody (Y12–259), and horseradish peroxidase-linked anti-rabbit, -mouse, and -goat antibodies were purchased from Santa Cruz Biotechnology. Dulbecco's modified Eagle's medium, fetal bovine serum, restriction enzymes, and the random primer DNA labeling kit were purchased from Life Technologies, Inc. All radioactive probes were obtained from NEN Life Science Products. Hoechst dye 33342 was obtained from Molecular Probes (Eugene, OR). XAR-5 film was obtained from Eastman Kodak.

**Preparation of Recombinant Adenovirus**—The recombinant adenovirus containing the cDNA encoding the p85N-SH2 domain of bovine p85α protein was isolated by homologous recombination using two plasmids, pACMVLPa (33) and pJM17 (34). The recombinant plasmids p85N-SH2 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 virus containing the p85N-SH2 domain of bovine p85α is denoted as Ad5-p85N-SH2, which is replication-defective (at least in cells lacking the E1 region of adenovirus), but fully infectious.

**Detection of Recombinant Ad5-p85N-SH2 or Wild-type by PCR**—DNA templates for PCR were extracted from the supernatant of the culture medium (350 μl) of the 293 cells that were infected with each plaque isolates/viruses at multiplicity of infection (m.o.i.) of 50 plaque-forming units (pfu)/ml. A multiplex PCR was performed on one-tenth of the 293 cells that were infected with each of the plaque isolates/adenoviruses at m.o.i. of 50. The multiplex PCR consisted of 50 μl of the following components: 1× PCR buffer (Promega, Madison, WI), 2.5 uM of each primer, and 1.25 units of Taq DNA polymerase (Boehringer Mannheim, Germany). The following primers were used: (1) p85N-SH2 forward primer, 5′-GCCGCGCCATGATACAAATAGTGGCTCAATTAATATATGT-3′; (2) p85N-SH2 reverse primer, 5′-GCCGCGCCATGATACAAATAGTGGCTCAATTAATATATGT-3′; (3) pJM17 forward primer, 5′-GCCGCGCCATGATACAAATAGTGGCTCAATTAATATATGT-3′; (4) pJM17 reverse primer, 5′-GCCGCGCCATGATACAAATAGTGGCTCAATTAATATATGT-3′; (5) p85N-SH2 forward primer, 5′-GCCGCGCCATGATACAAATAGTGGCTCAATTAATATATGT-3′; (6) p85N-SH2 reverse primer, 5′-GCCGCGCCATGATACAAATAGTGGCTCAATTAATATATGT-3′. The PCR products were analyzed by gel electrophoresis and detected by ethidium bromide staining. PCR products were sequenced to confirm their identity.

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viral incubation and were repeated three to five times. The efficiency of adenovirus mediated gene transfer was approximately 90% as measured by histocytotoxic staining for β-galactosidase (β-gal) of the cells infected with a recombinant adenovirus Ad5-lacZ, containing the bacterial β-galactosidase gene (data not shown). The survival of the different adenovirus constructs was unaffected by infection of the cells with the different adenovirus constructs since the total cell protein remained the same in infected or uninfected cells. However, the HIRc cells demonstrated about 10% decrease in total cellular protein, thereby indicating inhibition of cell cycle progression.

RNA Extraction and Northern Blot Analysis—Total RNA was isolated from the control (uninfected) and Ad5-p85N-SH2-infected cells as described elsewhere (36). Total RNA (15 μg) was denatured and electrophoresed on a 1% agarose, 6.6% formaldehyde gel and blotted onto a Hybond-N membrane. A 360-bp cDNA fragment of bovine p85SH2 domains was labeled with [α-32P]dCTP by random priming. Northern blot was hybridized with the labeled probe and washed as described previously (36). Ethidium bromide staining of the electrophoresed RNA was used to visualize the loading pattern. The autoradiograph was obtained after a 6-h exposure and analyzed with a desktop scanner (Hewlett Packard Scan Jet II P), utilizing a scan analysis program.

DNA Synthesis—Cells were infected with the indicated adenoviruses at m.o.i. of 2.0 and 10 for 1 h at room temperature, serum-starved for 36 h, and stimulated with insulin (100 ng/ml) in the presence of BrdUrd for 4 h. DNA synthesis was then measured by incorporation of BrdUrd into DNA as described elsewhere (36). Thirty μl of 2 M HCl was added to 60 μl of total protein with antibodies to IRS-1 or to phosphotyrosine residues (2–4 μg), overnight at 4 °C. Immune complexes were precipitated from the supernatant with protein A-Sepharose (Sigma) and washed as described (37). The washed immune complexes were incubated with phosphatidylinositol (Avanti) and [γ-32P]ATP (3000 Ciummol) for 10 min at room temperature. Reactions were stopped with 100 μl of 1 M EDTA, 50 mM Na3VO4, pH 7.4). The resulting cell extracts were centrifuged at 10,000 g for 10 min, washed with PBS, scraped, and frozen at −70 °C immediately. Frozen cell pellets were extracted in ice-cold RIPA buffer (50 mM Hepes, pH 7.4, 10 mM MgCl2, 150 mM NaCl, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of 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 each 2 μg of the anti-Ras antibody Y13–259 (Santa Cruz Biotechnology) or 2 μg of rat IgG (Cappel) were added. To both samples, goat anti-rat IgG and protein G-agarose were added as well as NaCl, sodium dodecyl sulfate (SDS), and deoxycholate to final concentrations of 500 mM, 0.05%, and 0.5%, respectively. The samples were shaken gently for 1 h at 4 °C and then the immunoprecipitates were washed four times in RIPA buffer containing 500 mM NaCl, 0.05% SDS, and 0.5% deoxycholate and two times in 20 mM TrisPO4, pH 7.4. The washed immunoprecipitates were resuspended in 30 μl of 5 mM TrisPO4, 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 GTP and GDP were measured as described below.

GTP was converted to ATP using NDP kinase and ADP with the ATP measured in the luciferase/luciferin system according to the following reaction.

**GTP + ADP → GDP + ATP**

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**GDP + pyrophosphate → GTP + pyrophosphate**

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 25 °C. **DNA synthesis** was determined as described previously (38), with some modifications. Confluent 3T3-L1 adipocytes (six-well culture dishes) were infected with the indicated adenovirus as described above for 2-deoxyglucose (2-DOG) assay, starved for 3 h in glucose and serum free DMEM containing 0.1% bovine serum albumin and 2 mM sodium pyruvate. Following this, the cells were either preincubated with 30 μM MEK inhibitor and stimulated with 200 ng/ml insulin for 30 min in DMEM containing 0.1% bovine serum albumin, 5 mM glucose, 2 mM sodium pyruvate. The cells were rinsed thrice with ice-cold PBS and then lysed in 150 μl of homogenization buffer (50 mM Tris-HCl, 10 mM EDTA, 100 mM KF (pH 7.9)) using a sonicator. Homogenates were centrifuged at 4,000 rpm for 10 min at 4 °C. Thirty μl of homogenate (equivalent to 10 μg of protein) was added to 60 μl of preheated glycogen synthase assay buffer (10% oyster glycogen, 10 μM uridine 5'-diphosphogluucose, 50 mM Tris-HCl, 25 mM Na2SO4, 50 mM NaF, 27 mM EDTA, 0.3 μCi of UDP-[14C]glucose per ml (pH 7.6) with and without 10 mM d-glucose 6-phosphate). The mixture was incubated at 30 °C for 30 min. Seventy-five microliters of each reaction mixture was applied to Whatman no. 4 filter paper. The filters were washed and dried and counted in scintillation fluid for counting. Glycogen synthase activation was calculated by subtracting glucose 6-phosphate-independent activity from glucose 6-phosphate-dependent activity.

**Ras GTP/GDP Assay—** Differentiated 3T3-L1 adipocytes were infected with Ad5-p85N-SH2 or Ad5-CMV at 50 m.o.i. for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 48 h. Following 24 h of serum starvation, the cells were stimulated with or without insulin (100 ng/ml) for 10 min, washed with PBS, scraped, and frozen at −70 °C immediately. Frozen cell pellets were extracted in ice-cold RIPA buffer (50 mM Hepes, pH 7.4, 10 mM MgCl2, 150 mM NaCl, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of 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 each 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, sodium dodecyl sulfate (SDS), and deoxycholate to final concentrations of 500 mM, 0.05%, and 0.5%, respectively. The samples were shaken gently for 1 h at 4 °C and then the immunoprecipitates were washed four times in RIPA buffer containing 500 mM NaCl, 0.05% SDS, and 0.5% deoxycholate and two times in 20 mM TrisPO4, pH 7.4. The washed immunoprecipitates were resuspended in 30 μl of 5 mM TrisPO4, 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 GTP and GDP were measured as described below.

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**GDP + pyrophosphate → GTP + pyrophosphate**

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 25 °C.
30 °C and contained in a final volume of 15 μl of 50 mM glycine, pH 7.8, 10 mM dithiothreitol, 8 mM MgSO4, 50 μM phosphoenolpyruvate, 3 milligrams 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 GTP + GDP to yield the amount of GDP. DNA was measured by a standard fluorescence method using the fluorescent dye bisbenzimidazole, 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 DNA or milligram protein in the cell lysate (40).

RESULTS

Detection of Recombinant Ad5-p85N-SH2 or Wild-type Virus by Multiplex PCR Analysis—Multiplex PCR amplification of the viral DNA was performed using E1A and E2B specific primers as described previously (31). The E1A DNA (indicative of wild-type virus) was not detected from any plaque-purified recombinant Ad5-p85N-SH2 (lanes 1–5) but was specifically detected when the DNA template from the wild-type infected cell culture was used as a template (lane 7). These results indicate that the recombinants were free of wild-type virus contamination. B (bottom panel), the recombinant Ad5 p85N-SH2 virus DNA (clones 1–5) were PCR-amplified with cDNA specific primers. A 386-bp fragment was detected in all the clones containing the recombinant Ad5 p85N-SH2 (lanes 2–6) but was not detected in the wild-type infected cell culture DNA (lane 7).

increase in mRNA levels of the p85N-SH2 domain of PI 3-kinase relative to the uninfected cells (lane 1).

The differentiated 3T3-L1 adipocytes were infected with the same clones of the p85N-SH2 expressing adenovirus at the indicated m.o.i. for 16 h and protein expression was examined 48 h later by Western blotting. Fig. 2B shows the results of an anti-Myc blot. Specific bands appeared at 18–20 kDa for the Myc-tagged p85N-SH2 protein in a viral dose-dependent manner.

Studies of HIRcB Fibroblasts: IRS-1-associated PI 3-Kinase Activity—It is well established that tyrosine phosphorylated IRS-1 associates with and activates PI 3-kinase (1, 5, 6). Therefore, we measured IRS-1-associated PI 3-kinase activity in p85N-SH2-overexpressing HIRcB cells. In theory, the p85-derived N-SH2 domain should competitively inhibit interactions between the SH2 domain of endogenous p85 and its protein partners. To assess this, whole cell lysates from insulin-treated HIRcB fibroblasts overexpressing p85N-SH2 proteins were subjected to immunoprecipitation with anti IRS-1 antibody and PI 3-kinase activity was determined in the precipitates. In control cells, insulin stimulation resulted in a 25–30-fold increase in IRS-1-associated PI 3-kinase (Fig. 3) and this effect was unchanged in Ad5-lacZ- or Ad5-CMV-infected cells (data not shown). Overexpression of increasing amounts of the p85N-SH2 domain led to a dose-dependent inhibition of IRS-1-associated PI 3-kinase activity (Fig. 3). These results are consistent with the hypothesis that the overexpressed p85N-SH2 protein behaves in a dominant negative manner, interfering with the interactions of endogenous p85 with phosphorylated IRS-1.

Effect of p85N-SH2 Domain on Insulin-stimulated DNA Synthesis—To evaluate a more distal biological effect, insulin-stimulated cell cycle progression was measured by monitoring BrdUrd incorporation into newly synthesized DNA. Cells were plated on coverslips and infected with Ad5-p85N-SH2, and Ad5-β-gal at m.o.i. of 2.0 and 10 pfu/cell, serum-starved for 36 h and then stimulated with insulin. As seen in Fig. 4, 45–55% of the uninfected cells underwent DNA synthesis in response to insulin and serum, respectively, and this response was not
Studies in 3T3-L1 Adipocytes—Effects of Overexpression of p85N-SH2 Domain on Insulin-stimulated PI3-kinase Activity in HIRcB Cells. Cells were infected with Ad5p85N-SH2 for 1 h at room temperature and grown in medium containing heat-inactivated serum (2%) for 48 h. Serum-starved (16 h) cells were incubated in the absence (basal) or presence of insulin (100 ng/ml) for 10 min, lysed, and subjected to immunoprecipitation with antibodies to IRS-1. The washed immunoprecipitates were assayed for PI 3-kinase activity with phosphatidylinositol as substrate, and the labeled PI 3-phosphate product (PI-3P) was resolved by thin-layer chromatography and visualized by autoradiography. This experiment was repeated four times with appropriate controls.

Noninfected cells (Fig. 5A). Thus, in control cells, insulin led to a 10–15-fold increase in IRS-1-associated PI 3-kinase activity, which was markedly inhibited by expression of the p85N-SH2 domain in a dose-dependent manner. At 50 m.o.i., the insulin-induced PI 3-kinase activity was almost completely abolished. In contrast, infection of the 3T3-L1 adipocytes with Ad5-CMV was without effect. Pretreatment of cells with 100 nM wortmannin for 30 min prior to insulin stimulation resulted in ~50% reduction in IRS-1-associated PI 3-kinase activity (data not shown).

We also assessed PI3-K activity associated with phosphorylase residues in anti-phosphorylase immunoprecipitates from control and p85N-SH2-infected 3T3-L1 adipocytes in the presence and absence of insulin. In control cells, insulin stimulation resulted in ~15-fold increase in phosphorylase-associated PI3-K activity, which was completely inhibited by expression of the p85N-SH2 domain (Fig. 5B).

p21ras GTP Loading—Since the relationship between PI 3-kinase and Ras is a matter of debate, we examined the effect of PI 3-kinase inhibition on the insulin-induced stimulation of p21ras in 3T3-L1 adipocytes. Stimulation of 3T3-L1 adipocytes, infected with a control adenovirus (Ad5-CMV) with 100 ng/ml insulin for 10 min resulted in a 2–3-fold stimulation of p21ras activation (Fig. 6). Surprisingly, unstimulated cells overexpressing the p85N-SH2 protein showed a 2–3-fold higher, basal p21ras-GTP activity, which is similar to the insulin-induced state. Stimulation of these cells with insulin for 10 min did not have any further effect on p21ras-GTP loading.

Insulin-stimulated Activation of MAPK—To investigate whether PI 3-kinase is required for the induction of MAPK...
activity, or alternatively, whether it plays an indirect role in regulating MAPK activation through its effect on Ras-GTP levels, we determined the activation state of MAPK in uninfected, control, or p85N-SH2-infected 3T3-L1 adipocytes in the presence and absence of insulin. Cell lysates were subjected to Western blot analysis with phosphospecific MAPK antibodies. This antibody detects the tyrosine-phosphorylated (position 204) activated form of p44 and p42 ERK1 and ERK2) MAPK. In uninfected cells, insulin stimulated phosphorylation of MAPK by about 20-fold (Fig. 7A, lane 2). Overexpression of the p85N-SH2 domain led to a dose-dependent inhibition of both p44 and p42 MAPK phosphorylation and activation (Fig. 7A, lanes 3–6). Expression of MAPK was assessed by Western blotting with a polyclonal MAPK antiserum that recognizes both nonphosphorylated and phosphorylated forms and protein levels were not altered by infection with either the p85N-SH2 domain or Ctrl adenoviruses (Fig. 7B, lanes 1–6).

Insulin-stimulated Activation of p70S6K and AKT—It has been shown that p70S6K is downstream of PI 3-kinase, and activation of this enzyme was determined by Western blot analysis of whole cell lysates with a phosphospecific p70S6K antibody that detects p70S6K only when phosphorylated at Thr-421/Ser-424 and does not cross-react with other phosphoforms of p70S6K. This antibody also recognized glycogen synthase kinase 3β (GSK-3β). Phosphorylation of GSK-3β was not significantly altered by insulin. In uninfected cells, insulin increased phosphorylation of p70S6K by about 8-fold in uninfected cells (Fig. 9A, lanes 1 and 2), stimulation of 3T3-L1 adipocytes with insulin resulted in activation of p70 S6 kinase, which was inhibited by pretreatment with rapamycin (Fig. 8A, upper panel, lane 1). The p85N-SH2-overexpressing cells demonstrated a 60–90% inhibition of insulin-induced p70S6K activation at 10 and 50 m.o.i., respectively (Fig. 8A, upper panel, lanes 5 and 7). Similar results were obtained when p70S6K activation was assessed by retarded migration on SDS-PAGE using anti-p70S6K antibody. As shown in Fig. 8A (lower panel, lane 2), stimulation of cells with insulin resulted in activation of p70S6K, which was blocked by p85N-SH2 expression (Fig. 8A, lower panel, lanes 5 and 7). AKT (PKB) is also activated by insulin in a PI 3-kinase-dependent manner, and we assessed the degree of AKT activation in cell lysates, derived from insulin-stimulated 3T3-L1 cells as determined by retarded migration on SDS-PAGE (Fig. 8B, lane 2). Insulin treatment stimulated an AKT mobility shift, which was inhibited by wortmannin or p85N-SH2 expression (Fig. 8B, lanes 1, 5, and 7, respectively). These findings suggest that IRS-1-associated PI 3-kinase activity is necessary for insulin stimulation of p70S6K and for AKT activation.

Insulin-stimulated Glucose Transport—Cells were infected with Ad5-p85N-SH2, or Ad5-CMV and 2-DG uptake was measured 48 h later. As seen in Fig. 9A, insulin led to an 8-fold increase in 2-DG uptake, and this was not affected by Ad5-CMV infection. p85N-SH2 expression at 20 and 50 m.o.i. markedly inhibited insulin-stimulated 2-DG uptake. Expression of the p85N-SH2 also led to a slight decline in the basal 2-DG. Viral mediated expression of the unrelated Shc SH2 domain had no effect on basal or insulin-stimulated glucose uptake (data not shown). To further evaluate this finding, additional experiment was performed in which insulin dose-response studies were carried out in control, Ad5-p85N-SH2-, and Ad5-CMV-infected cells. As shown in Fig. 9B, insulin-mediated glucose transport was inhibited by p85N-SH2, while control adenovirus was without effect.

Co-expression of p110CAAX Rescues 2-DG Uptake Inhibited by the p85N-SH2 Protein—The effect of coexpression of p85N-SH2 domain and a membrane-targeted, p110 subunit of PI 3-kinase (p110CAAX) on 2-deoxyglucose uptake in 3T3-L1 adipocytes is shown in Fig. 9C. Insulin stimulated glucose transport about 8-fold in uninfected cells (Fig. 9C). Overexpression of a membrane-targeted (by C-terminal prenylation), p110 subunit of PI 3-kinase (p110CAAX) stimulated glucose transport to the same extent as insulin, and addition of insulin had no further effect. The p85N-SH2 blocks insulin induced 2DG uptake; however, co-expression of p110CAAX with p85N-SH2 rescued 2-DG uptake by ~90% without any added insulin.

Glycogen Synthase Activity—Fig. 10 shows glycogen synthase activity in differentiated 3T3-L1 adipocytes. Glycogen 6-phosphate-independent activity is expressed as a percentage of total glycogen synthase activity. In both nontransfected (not shown) and Ad5-CMV (control)-infected cells, insulin stimulated glycogen synthase activity ~3-fold. Preincubation of cells with 30 μM MEK inhibitor (PD 98059) had no effect on insulin induced glycogen synthase activity. The p85N-SH2 inhibited glycogen synthase activity by about 50%. These data suggest that activation of PI 3-kinase is necessary for activation of

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Multiple Roles of PI 3-Kinase

Insulin stimulation leads to extensive tyrosine phosphorylation of IRS-1. Phosphorylated IRS-1 binds to the SH2 domains of the p85 subunit of PI 3-kinase and activates its p110 subunit, and this is a major mechanism by which insulin stimulates this enzyme. Several lines of evidence suggest that PI 3-kinase is involved in mediating mitogenic effects of various growth factors (2, 5, 42) and is a necessary molecule for insulin stimulation of glucose transport (18–23). Despite a great deal of information on both the structure and function of PI 3-kinase, the precise mechanism of its involvement in mediating insulin signaling is not fully understood.

In the current study, we have extensively evaluated the role of PI 3-kinase activity in a diverse array of insulin actions. Our approach is based on the hypothesis that cellular overexpression of the N-SH2 domain of the p85 subunit would competitively inhibit complex formation between endogenous PI 3-kinase and its SH2 domain binding targets such as IRS-1, -2, -3, -4, the insulin receptor, and others. This in turn, will prevent insulin activation of PI 3-kinase enzymatic activity, allowing us to elucidate the biologic actions of this enzyme in insulin target cells such as 3T3-L1 adipocytes. Indeed our data show not only a marked inhibition of insulin-stimulated IRS-1-associated PI 3-kinase activity, but we find a complete blockade of insulin-stimulated anti-phosphotyrosine-associated PI 3-kinase activity, which would include the non-IRS-1-associated activity. Predictably, and in agreement with previous findings, inhibition of PI 3-kinase activity resulted in abrogation of insulin's effects on glucose uptake and DNA synthesis (12–17). Other major findings of these experiments are that inhibition of PI 3-kinase activity resulted in abrogation of glucose uptake and DNA synthesis (12–17). Other major findings of these experiments are that inhibition of PI 3-kinase activity resulted in inhibition of glycogen synthase, whereas MAPK does not appear to be involved.

DISCUSSION

The activation of MAPK by insulin depends on activation of Ras (1, 13). Tyrosine-phosphorylated Shc (12), and to a lesser extent IRS-1 (1, 5, 6), binds to the adaptor protein, Grb-2, which is preassociated with the guanine nucleotide exchange factor, Sos, that promotes the formation of the active GTP-bound state of Ras. Formation of GTP-bound Ras leads to activation of the protein serine/threonine kinase cascade including Raf-1 kinase, MEK and MAPK (1, 13). Recent studies using specific inhibitors of PI 3-kinase or constitutively active and dominant negative mutants of the enzyme, have yielded confusing, and potentially conflicting results suggesting that PI 3-kinase can be either downstream or upstream of Ras (26–30). In the present study, using 3T3-L1 adipocytes, we have demonstrated that inhibition of PI 3-kinase decreased insulin-induced activation of MAPK, although the levels of GTP-bound Ras were increased 2-fold in the basal state. Thus, PI 3-kinase may be required for the activation of MAPK at a step independent of and downstream of Ras.

The importance of PI 3-kinase for activation of ERKs 1 and 2 has been controversial. Expression of activated forms of p110α has been reported to stimulate the MAPK pathway in one case (29), but not in others (32, 43–45). A recent study found that overexpression of the p110y isoform resulted in activation of ERKs 1 and 2, whereas p110α was without effect (45). Our

FIG. 8. A, effects of overexpression of the Ad5-p85N-SH2 protein on insulin-stimulated p70S6K activation in 3T3-L1 adipocytes. Cells were infected with Ad5-p85N-SH2 expressing adenovirus at 10 and 50 m.o.i. for 12–16 h. Serum-starved (16 h) cells were incubated in the absence (basal) or presence of insulin (100 ng/ml) for 30 min, lysed, and subjected to SDS-PAGE and immunoblotted with phospho-specific p70 S6 kinase antibody (upper panel). The above filter was stripped and rebotted with p70 S6 kinase antibody. Insulin stimulation of serine phosphorylation of p70 S6 kinase is detected by a retarded migration of the enzyme (lower panel, lane 2). Preincubation with 100 nm rapamycin for 30 min inhibited activation of p70 S6 kinase (lower panel, lane 1). Similarly, overexpression of PTB and SAIN proteins inhibited p70 S6 kinase activation in a dose-dependent manner (lower panel, lanes 4–7). This experiment was repeated twice. B, effects of overexpression of Ad5-p85N-SH2 protein on insulin-stimulated AKT activation in 3T3-L1 adipocytes. Whole cell lysates (60 μg) were prepared as described in A, were subjected to SDS-PAGE and immunoblotted with AKT antibody. Insulin stimulation of serine phosphorylation of AKT is detected by a retarded migration of the enzyme (lane 2). Preincubation with 100 nm wortmannin for 30 min inhibited activation of AKT (lane 1). Overexpression of the p85N-SH2 protein (lanes 4–7) inhibited insulin-induced AKT activation (lanes 5 and 7). This experiment was repeated three times. Ctrl, control.
Differentiated 3T3-L1 adipocytes were infected with increasing m.o.i. of Ad5-p85N-SH2 and Ad5-p110C for 16 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 48 h. The cells were glucose- and serum-starved in DMEM containing 0.1% bovine serum albumin, 2 mM sodium pyruvate for 2.5 h, then incubated with or without 50 μM MEK inhibitor for 30 min, following which cells were stimulated with 200 ng/ml insulin for 30 min in 5 mM glucose-containing medium. After 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 (G-6-P). Results are expressed as mean ± S.E. of percentage of glycogen synthase index (% GSI) from three independent experiments. Ctrl, control.

Findings indicating the involvement of PI 3-kinase in the activation of MAPK seem to be consistent with a recent report showing that treatment of 3T3-L1 adipocytes, either with wortmannin or LY294002, inhibited insulin-induced activation of Raf-1 and MAPKs with no effect on the formation of GTP-bound Ras (46). However, wortmannin did not affect epidermal growth factor-induced activation of Raf and MAPKs in the same system, indicating that differential mechanisms to activate Raf-1 and MAPKs by insulin and epidermal growth factor exist in 3T3-L1 adipocytes. Very similar observations have been reported using rat adipocytes (47). However, the involvement of PI 3-kinase in MAPK activation appears to be cell type-specific, because wortmannin did not inhibit insulin-induced activation of MAPK in CHO cells in the above study (46).

As mentioned earlier, the relationship between PI 3-kinase and Ras is a matter of debate. Some observations indicate that PI 3-kinase could be upstream, downstream, or independent of Ras; these observations are perhaps related to cell-type differences (reviewed in Ref. 48). Our data reinforce the idea that PI 3-kinase is upstream and can activate the Ras pathway. However, our unexpected findings were the influence of overexpression of the p85N-SH2 domain of PI 3-kinase to stimulate formation of GTP-z-GTP in 3T3-L1 adipocytes. PI 3-kinase and Ras form a complex, suggesting an intimate relation between the function of these molecules (26), and it can be argued that a loss of PI 3-kinase activity could disrupt basal Ras-p110 binding, which may be required for PI 3-kinase signaling.

It is known that insulin stimulates p21ras-GTP loading by increasing the guanine nucleotide exchange activity (GDP-GTP) of Sos (49, 50). Return of p21ras into its inactive conformation is facilitated by the idea that PI 3-kinase is upstream and can activate the Ras pathway. However, our unexpected findings were the influence of overexpression of the p85N-SH2 domain of PI 3-kinase to stimulate formation of p21ras-GTP in 3T3-L1 adipocytes. PI 3-kinase and Ras form a complex, suggesting an intimate relation between the function of these molecules (26), and it can be argued that a loss of PI 3-kinase activity could disrupt basal Ras-p110 binding, which may be required for PI 3-kinase signaling.

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by GAP that hydrolyzes GTP to GDP. Therefore, overexpression of the p85N-SH2 domain of PI 3-kinase can stimulate formation of p21GTP either by stimulating the guanine exchange activity of Sos or by diminishing the activity of GAP. Stimulation of Sos activity is unlikely because Langolis et al. (51) have shown that immunodepletion of PI 3-kinase from lysates of insulin-stimulated cells does not alter the activity level of the guanine nucleotide exchange factor Sos. Therefore, the most likely explanation for the increase in basal GTP-bound Ras in the p85N-SH2 expressing cells is that the p85N-SH2 depletes the pool of GAP in these cells. Indeed, in 3T3-L1 adipocytes, the p85 subunit of PI 3-kinase is already in excess compared with the p110 subunit, and it has been shown to interact with GAP (presumably via p62, a GAP-associated protein) (52). Overexpression of the p85N-SH2 peptide could tritate out cellular GAP by direct association, such that GAP is not available to hydrolyze p21GTP. The elevated p21GTP levels do not appear to affect MAPK activity, since we find that the p85N-SH2 protein inhibits insulin-induced MAPK phosphorylation in 3T3-L1 adipocytes. Therefore, the inhibitory effect of PI 3-kinase on the MAPK cascade most likely occurs at a level independent of Ras. Therefore, in these cells, insulin is able to stimulate MAPK via a Ras-independent pathway. These results are in agreement with the observations of Carel et al. (53), who also found that insulin activates MAPK by a Ras-independent pathway in 3T3-L1 adipocytes and by a Ras-dependent pathway in 3T3-L1 fibroblasts.

In an earlier report, we utilized an adenovirus system to express the PTB domain of IRS-1 in 3T3-L1 adipocytes. This led to a marked decrease in IRS-1-associated PI 3-kinase activity, but no inhibition of insulin-stimulated glucose transport (31). The current study further extends our earlier proposed hypothesis that complete blockade of PI 3-kinase activity (i.e., IRS-1 and the non-IRS-1-associated components) would be necessary to inhibit insulin-induced glucose transport. Indeed, in these cells, insulin is able to stimulate MAPK via a Ras-independent pathway. These results are in agreement with the observations of Carel et al. (53), who also found that insulin activates MAPK by a Ras-independent pathway in 3T3-L1 adipocytes and by a Ras-dependent pathway in 3T3-L1 fibroblasts.

We also examined additional targets of insulin action which are thought to be downstream of PI 3-kinase, to further explore the effects of PI 3-kinase inhibition. Akt, a serine/threonine kinase, is a downstream target of PI 3-kinase and is activated by a dual mechanism involving the binding of phosphatidylinositol 3,4-bisphosphate to its PH domain, as well as serine/threonine phosphorylation by one or more Akt kinases, which may themselves be stimulated by the lipid products of PI 3-kinase (56). Expression of a constitutively active Akt in rat adipocytes (57) and 3T3-L1 adipocytes (58) increases glucose uptake and GLUT4 translocation. Consistent with these findings, our data show that Akt activation is dependent on PI 3-kinase activity, since we find that pretreatment of 3T3-L1 adipocytes with wortmannin, a PI 3-kinase inhibitor, as well as overexpression of the dominant inhibitory p85N-SH2 protein, leads to an inhibition of insulin-induced serine/threonine kinase activity of Akt. In addition, insulin-induced glucose transport and glycogen synthase were inhibited when 3T3-L1 adipocytes were pretreated with wortmannin, or when the p85N-SH2 protein was overexpressed. Similarly, insulin-induced phosphorylation of another downstream target of PI 3-kinase, p70 S6 kinase, was inhibited by Rapamycin as well as by the dominant inhibitory p85N-SH2 protein. These results suggest a role for Akt as a PI 3-kinase-dependent upstream activator of glucose transport and/or a role for p70 S6 kinase as a PI 3-kinase-dependent upstream activator of glycogen synthase activity.

Our data on expression of the membrane-targeted p110CAAX indicate that membrane localization of PI 3-kinase is critical for biologic functions, since this form of the p110 subunit leads to stimulation of 2-deoxyglucose transport similar to the insulin-induced levels in 3T3-L1 adipocytes. Along similar lines, it has been shown in COS-7 cells, that targeting p110 to the membrane, either by N-terminal myristoylation or by C-terminal farnesylation, can stimulate p70 S6 kinase and Akt activity in the absence of insulin. Our results on co-expression of the p110CAAX with the N-SH2 domain strongly support the idea that the p85N-SH2 protein specifically blocks insulin-mediated PI 3-kinase activity, by competitively interfering with the targeting of p85 to its natural protein partners. Thus, although the functional activity of downstream signaling molecules, such as p70 S6 kinase, Akt, and GLUT4, are functionally blocked by expression of the p85N-SH2, the fact that the biologic activity can be rescued by co-expression of the N-SH2 domain with p110CAAX demonstrates that the signaling pathways downstream of p85 targeting are intact and that the dominant/negative effects of the p85N-SH2 domain do not reflect nonspecific actions at other cellular loci.

Our studies also show that overexpression of the p85N-SH2 domain markedly inhibits insulin-stimulated glycogen synthase activity. This leads to the conclusion that PI 3-kinase stimulation is necessary for insulin activation of glycogen synthase. The molecular mechanisms by which glycogen synthase is regulated by insulin remains one of the crucial issues in insulin action. Insulin stimulates glycogen synthesis via a coordinated response involving activation of protein phosphatase 1, by phosphorylation of its G subunit and by a phosphorylation-induced inactivation of glycogen synthase kinase 3 (GSK3) (59, 60). It has been suggested that p1 might be downstream of the MAPK pathway, but a number of reports have indicated that this is not the case (61). In addition, our own results show that the MEK inhibitor (PD098059) did not lead to a decrease in insulin stimulation of glycogen synthesis. Thus, it is unlikely that any effect of the p85N-SH2 protein on the MAPK pathway is mediating the inhibitory effect on glycogen synthesis. It has been reported that GSK3 is a downstream target of Akt, which, in turn, is dependent on PI 3-kinase activity (62). Thus, one potential interpretation of our results is that inhibition of PI 3-kinase, through the dominant/negative p85N-SH2 domain, prevents insulin-stimulated GSK3 phosphorylation, and that this leads to inhibition of glycogen synthesis. However, it has recently been shown that GSK3 expression is either very low, or not expressed, in 3T3-L1 adipocytes (63–65), making this possibility less likely. Thus, although the exact mechanism is unknown, our results clearly show that insulin-stimulated glycogen synthesis is dependent on proper targeting of PI 3-ki-
nase, and this is consistent with other reports showing that insulin-stimulated glycogen synthesis can be inhibited by wortmannin or LY294002 (21, 66), both of which are relatively specific inhibitors of PI 3-kinase activity.

In conclusion, we have shown that overexpression of the p85N-SH2 domain in 3T3-L1 adipocytes inhibits a variety of insulin’s ultimate biologic effects, and that this domain will also block insulin-stimulated DNA synthesis in fibroblasts. These results are consistent with our earlier findings, which suggested that a complete blockade of PI 3-kinase targeting to all of its cellular substrates (IRS-1 as well as non-IRS-1 targets) would be necessary for inhibition of AKT activation and glucose transport stimulation. We also find that in 3T3-L1 adipocytes, PI 3-kinase can regulate p110α-GTP levels and that the dominant-negative effects of the p85NSH2 domain on the MAP kinase pathway are exerted distal to the stimulatory effect of p110α-GTP on the MAPK pathway.

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