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Functional Interactions of Phosphatidylinositol 3-Kinase with GTPase-Activating Protein in 3T3-L1 Adipocytes

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The role of phosphatidylinositol (PI) 3-kinase in specific aspects of insulin signaling was explored in 3T3-L1 adipocytes. Inhibition of PI 3-kinase activity by LY294002 or wortmannin significantly enhanced basal and insulin-stimulated GTPase-activating protein (GAP) activity in 3T3-L1 adipocytes. Furthermore, removal of the inhibitory influence of PI 3-kinase on GAP resulted in dose-dependent decreases in the ability of insulin to stimulate $p21^{ras}$. This effect was specific to adipocytes, as inhibition of PI 3-kinase did not influence GAP in either 3T3-L1 fibroblasts, Rat-1 fibroblasts, or CHO cells. Immunodepletion of either of the two subunits of the PI 3-kinase (p85 or p110) yielded similar activation of GAP, suggesting that catalytic activity of p110 plays an important role in controlling GAP activity in 3T3-L1 adipocytes. Inhibition of PI 3-kinase activity in 3T3-L1 adipocytes resulted in abrogation of insulin-stimulated glucose uptake and thymidine incorporation. In contrast, effects of insulin on glycogen synthase and mitogen-activated protein kinase activity were inhibited only at higher concentrations of LY294002. It appears that in adipocytes, PI 3-kinase prevents activation of GAP. Inhibition of PI 3-kinase activity or immunodepletion of either one of its subunits results in activation of GAP and decrease in GTP loading of $p21^{ras}$.

Insulin binding to its cell surface receptor initiates diverse metabolic and mitogenic signals that reach their final destination via an elaborate network of intracellular signaling molecules (5, 52). The precise intracellular routes of specific signals responsible for defined aspects of insulin action are not fully understood. It is assumed, however, that the signals may merge and diverge at the levels of several key intermediates such as insulin receptor substrate 1 (IRS-1), phosphatidylinositol (PI) 3-kinase, $p21^{ras}$, mitogen-activated protein (MAP) kinases, etc., creating an elaborate network of signal transduction. Although the mechanisms and significance of reciprocal interactions among multiple signaling intermediates are being explored in many laboratories, numerous aspects of these interactions remain enigmatic.

The complexity of interactions in the network of insulin signaling can be illustrated by an interplay between the Ras pathway and signals traveling via the PI 3-kinase-dependent path. Insulin has been shown to activate PI 3-kinase and $p21^{ras}$ in many types of cells (2, 23, 36, 38, 51, 54). Activation of both appears to be crucial for the mitogenic effects of insulin (7, 24). In contrast, metabolic signals of insulin appear to segregate between the Ras and the PI 3-kinase pathways. Insulin-stimulated glucose uptake has been shown to be dependent on activation of PI 3-kinase (6, 41), whereas stimulation of MAP kinase and glycogen synthase seems to be related to the Ras pathway (47). The issue of cross talk between these two pathways in insulin signaling has not been explored in detail. Yamauchi et al. (65) have demonstrated that PI 3-kinase lies upstream from Ras and Raf in mediating mitogenic effects of insulin. Experiments using wortmannin to inhibit PI 3-kinase activity indicated that an effect of insulin on $p21^{ras} \cdot$ GTP loading in CHO cells was independent of PI 3-kinase (19). Recently, however, a reciprocal relationship between PI 3-kinase and $p21^{ras}$ has been demonstrated (22, 49). Thus, $p21^{ras}$ has been shown to stimulate PI 3-kinase by one group of investigators (49), whereas another group has demonstrated that some cellular responses to constitutively active PI 3-kinase are Ras dependent (22).

Since most of the data available to date were generated in diverse types of cells (some of which are not physiologic targets of insulin action), in this study, we investigated the interaction of PI 3-kinase with $p21^{ras}$ following insulin stimulation in 3T3-L1 adipocytes. It appears that in 3T3-L1 adipocytes, PI 3-kinase inhibits GTPase-activating protein (GAP), allowing the insulin signal to fully activate $p21^{ras}$ via stimulation of guanine nucleotide exchange activity of Sos.

MATERIALS AND METHODS

Methods. Cell culture media and supplies were from GIBCO-BRL (Gaithersburg, Md.) and Gemini Bioproducts (Calabasas, Calif.); radioisotopes were from New England Nuclear (Billerica, Mass.). All standard chemicals were from Sigma (St. Louis, Mo.). Insulin and LY294002 were kindly provided by Eli Lilly & Co. (Indianapolis, Ind.). Anti-PI 3-kinase p85 polyclonal antibody was from Transduction Laboratories (Lexington, Ky.), p110 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, Calif.), and GAP polyclonal antibody was from Upstate Biotechnology Incorporated (Lake Placid, N.Y.). Wortmannin was from Brimol (Plymouth Meeting, Pa.), and Ha-Ras was a gift from A. Wolfman, Cleveland Clinic Foundation (Cleveland, Ohio).

Cell culture. CHO and Rat-1 fibroblasts transfected with wild-type human insulin receptors (CHO-IR and HIRc with approximately 3×10^5 insulin receptors per cell) were grown to confluence in Dulbecco's modified Eagle's medium-F12 with 10% fetal calf serum (FCS), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 50 μg of gentamicin per ml, and 500 nM methotrexate. 3T3-L1 fibroblasts were grown to confluence in fibroblast growth medium (Dulbecco's modified Eagle's medium–5.5 mM glucose with 10% FCS, 50 μg of gentamicin per ml, and 0.5 mM glutamine). Differentiation of 3T3-L1 fibroblasts into adipocytes was induced via the following protocol. Two days after confluency, cells were fed differentiation medium (Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% FCS, 50 μg of gentamicin per ml, and 0.5 mmol and glutamine) plus differentiation mix (2.5 ml of 10× phosphate-buffered saline [PBS], 0.055 g of isobutylmethylxanthene, 20 ml of deionized water, 250 μl of 49 mM dexamethasone, 2.5 mg of insulin). On day 4, cells were fed adipocyte growth medium (Dulbecco's modified Eagle's medium containing 25 mM glu-

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cose with 10% FCS, 50 μg of gentamicin per ml, 0.5 mM glutamine, and 1 μg of insulin per ml). Cells were refed these media without insulin every 2 days and used on days 10 to 12.

Insulin-mediated $p21^{ras} \cdot GTP$ formation. Confluent CHO-IR, HIRc, or 3T3-L1 fibroblasts and adipocytes were serum and phosphate starved for 24 h and labeled with $^{32}P_i$ (0.25 mCi/ml) overnight. Cells were then incubated first with LY294002 (0 to 100 μM) or wortmannin (0 to 100 μM) for 30 min and then with 100 nM insulin for 10 min. The increment in percent $p21^{ras}$ was determined as previously described (12), with minor modifications. Briefly, precleared lysates were immunoprecipitated with anti-Ras antibody Y13-259 (100 ng/ml; Oncogene Science, Cambridge, Mass.), and the nucleotides were separated by thin-layer chromatography. GTP and GDP were visualized by autoradiography and by using acid molybate reagent, cut, and quantified by liquid scintillation counting.

GAP activity. Determinations of GAP activity were performed as previously described (12), with minor modifications. In brief, cell lysates were prepared from 3T3-L1 adipocytes either untreated or pretreated with LY294002 (0 to 50 μM) for 30 min and then incubated with or without 100 nM insulin for 10 min. To measure GAP activity, c-Ha-Ras (367 ng) was incubated with 6.7 μM [γ-^{32}P] GTP (200 μCi/ml) and 745 ng of chain A insulin per ml in low-Mg^{2+} binding buffer (25 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 400 ng of bovine serum albumin per ml) at 30°C for 15 min. The GTP loading reaction was stopped with addition of excess free Mg^{2+}. Ras · GTP complex (250 μl, 50 ng) was added to 50 μl of lysate (50 μg of protein) or lysis buffer and incubated at 23°C. At intervals (0 to 15 min), aliquots were removed, filtered through a 0.45-μm-pore-size Millipore nitrocellulose filter, and washed three times with 1 ml of ice-cold buffer. The GAP activity of cell lysates was determined by measuring the loss of protein-bound radioactivity by liquid scintillation counting. The results are expressed as percent hydrolyzed GTP. A value of 100% remaining represents 20,051 ± 1,069 cpm bound. Background counts per minutes were less than 2% of total bound [^{32}P]GTP.

Thymidine incorporation. Confluent cells were serum starved for 24 h and then treated with insulin (0 to 100 nM) and with or without LY294002 (50 μM) for 15 h. [^{3}H]thymidine was added for the final 3 h. Cells were washed three times with PBS and precipitated with trichloroacetic acid. Precipitable counts were solubilized and counted with a scintillation counter (13).

Glycogen synthase activity. Confluent 3T3-L1 adipocytes were serum starved and incubated overnight with insulin and/or LY294002 (0 to 10 μM). The cells were rinsed twice with ice-cold PBS and then homogenized in 0.5 ml of homogenization buffer (0.7 M sucrose, 111 mM EDTA, 87.5 mM NaF, 35 mM Tris-HCl [pH 8]). Homogenates were centrifuged at 14,000 × *g* for 20 min at 4°C. Fifty microliters of homogenate was added to 50 μl of preheated glycogen synthase assay buffer (10% oyster glycogen, 10 mM uridine 5′-diphosphoglucose, 50 mM Tris-HCl, 25 mM Na_2SO_4, 50 mM NaF, 27 mM EDTA, 0.3 μCi of UDP-[^{14}C]glucose per ml [pH 7.6] with and without 10 mM D-glucose-6-phosphate). The mixture was incubated at 30°C for 20 min. Fifty microliters of each reaction mixture was applied to Whatman no. 4 filter paper. The filters were air dried and washed three times for 40 min each in 66% ethanol. The final wash was with 100% acetone. Filters were dried and placed in scintillation fluid for counting. Glycogen synthase activation was calculated by subtracting glucose-6-phosphate-independent activity from glucose-6-phosphate-dependent activity.

Glucose uptake. Confluent 3T3-L1 cells were refed with serum-free medium and pretreated with LY294002 (0 to 10 μM) for 30 min prior to use. Cells were rinsed and incubated in Krebs Ringer bicarbonate HEPES containing 1% bovine serum albumin (1% KRHB) with and without insulin (100 nM) and LY294002. Glucose uptake was assessed by a 5-min pulse with 0.5 μCi of [^{3}H]2-deoxyglucose per plate to a final glucose concentration 0.1 mM. Cells were washed three times with ice-cold PBS and solubilized in 1 NaOH, and ^{3}H counts were assessed by scintillation counting. Under these conditions, simple diffusion measured by L-glucose uptake was less than 5% of 2-deoxyglucose uptake (48).

MAP kinase activity. MAP kinase activity was determined as described by Heasley et al. (20). Confluent serum-starved 3T3-L1 adipocytes were incubated with LY294002 (0 to 50 μM) for 30 min, challenged with 100 nM insulin for 10 min, rinsed, and lysed with MAP kinase lysis buffer (0.5% Triton X-100, 50 mM β-glycerol phosphate, 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA, 5 μg of leupeptin per ml, 0.2 U of aprotinin per ml [pH 7.2]). Cell debris were removed by centrifugation at 10,000 × *g* for 5 min, and then cell extracts were adsorbed to 0.5-ml DEAE-Sephacel (Pharmacia, Piscataway, N.J.) columns, washed with three 1-ml aliquots of 40 mM β-glycerophosphate P–0.1 mM sodium vanadate–1 mM dithiothreitol–1 mM EGTA (pH 7.2), and then eluted with the same buffer containing 500 mM NaCl. Aliquots (20 μl) of column eluant were incubated for 10 min at 30°C with 20 μl of 50 mM β-glycerophosphate–0.1 mM sodium vanadate–1 mM $MgCl_2$–1 mM EGTA (pH 7.2)–100 μM [γ-^{32}P]ATP (5,000 cpm/pmol)–25 μg of cyclic AMP-dependent protein kinase inhibitor (PKI 5-24, Bachem, Torrance, Calif.) per ml–20 μM $EGFR_{662\text{-}681}$ peptide (RRELVEPLTPSGEAPNQALLR). The reactions were terminated by the addition of 10 μl of 25% (wt/vol) trichloroacetic acid, and aliquots were spotted on Whatman P-81 phosphocellulose (Fisher Scientific, Pittsburgh, Pa.) and washed with 75 mM phosphoric acid four times. Phosphorylated $EGFR_{662\text{-}681}$ peptide retained on filters was quantified by scintillation counting.

Immunoprecipitation of p85 and p110. Confluent 3T3-L1 adipocytes were serum starved for 24 h, then challenged with 100 nM insulin for 15 min, rinsed, and lysed with buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40). Cell debris were removed by centrifugation 10,000 × *g* for 5 min. Lysates were precleared with protein G-protein A-agarose (Oncogene Science, Cambridge, Mass.) precoupled to immunoglobulin G antibody for 30 min at 4°C. Precleared lysates were immunoprecipitated with either 4 μg of anti-PI 3-kinase (anti-p85) polyclonal antibody or 1 μg of PI 3-kinase p110 polyclonal antibody for 1 h at 4°C, then precipitated with 5 μg of rabbit anti-mouse immunoglobulin antibody, and rotated for an additional 30 min at 4°C. Protein G-protein A-agarose was added to the immunoprecipitation complex, which was then incubated for an additional 30 min at 4°C, centrifuged at 15,000 × *g* for 10 min, washed, and lyophilized to dryness. In some experiments, postimmunoprecipitation lysates were used to measure GAP activity as described above.

Western blotting (immunoblotting) for GAP. Immunoprecipitates were prepared as described above. Pellets were resuspended in 30 μl of 2× Laemmli buffer, boiled, loaded onto SDS–7.5% polyacrylamide gels, and subjected to polyacrylamide gel electrophoresis (PAGE) at 100 V for 90 min. Gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, Calif.), blocked with 5% milk, and incubated with either p85 (10 ml of Tris balanced salt Tween buffer [TBST]–1% milk with 10 μg of p85 polyclonal antibody [Transduction Laboratories]), p110 (10 ml of TBST–1% milk with 1 μg of p110 polyclonal antibody [Santa Cruz Laboratories]), or GAP (10 ml of TBST–1% milk with 20 μg of anti-human rat polyclonal GAP [Upstate Biotechnology]) primary antibodies for 1 h at room temperature.

Membranes were washed three times with TBST and incubated with secondary antibody solution (10 ml of TBST–1% milk with 10 μl of rabbit anti-immunoglobulin G (polyclonal antibody) (ECL Western Blotting Analysis System; Amersham Life Science) for 1 h at room temperature. Membranes were then washed five times with TBST and analyzed by chemiluminescence.

Statistics. All statistics were analyzed by Student's *t* test, with a *P* value of <0.05 considered significant. Results are expressed as means ± standard errors of the means (SEM) in comparison with values for control groups.

RESULTS

Previous experiments from this and other laboratories demonstrated that interruption of the signaling along the Ras pathway inhibited insulin effects on glycogen synthase and thymidine incorporation but did not affect glucose transport (6, 41, 47). In contrast, inhibition of the PI 3-kinase activity resulted in abrogation of the insulin effect on glucose uptake (in 3T3-L1 adipocytes), with no effect on glycogen synthase (in CHO cells) (19).

To attain a meaningful comparison of the roles of PI 3-kinase in mediating various metabolic effects of insulin, we first examined the influence of inhibition of PI 3-kinase activity on both glucose uptake and glycogen synthase activity in the insulin-responsive 3T3-L1 adipocytes. PI 3-kinase activity was blocked by a specific inhibitor of this enzyme, LY294002, a competitive inhibitor of the ATP-binding site (61). In agreement with results obtained by others (6), inhibition of PI 3-kinase activity effectively blocked both insulin-stimulated glucose uptake and thymidine incorporation (Fig. 1A and B). LY294002 also significantly reduced insulin-stimulated MAP kinase activity (Fig. 1C), but the effect of insulin on glycogen synthase was inhibited only at higher concentrations of LY294002 (Fig. 1D).

Since the activation of the MAP kinase and glycogen synthase by insulin has been linked to the Ras pathway (47), we examined the effect of PI 3-kinase inhibition on the insulin-induced stimulation of $p21^{ras}$. Surprisingly, inhibition of PI 3-kinase in 3T3-L1 adipocytes (10 to 12 days postdifferentiation) resulted in a marked and a dose-dependent inhibition of the insulin-stimulated $p21^{ras} \cdot GTP$ formation (Fig. 2). Similar inhibition of $p21^{ras}$ activation by insulin was observed in experiments with a structurally dissimilar PI 3-kinase inhibitor, wortmannin (Fig. 3). This effect appears to be specific for adipocytes, since there was no effect of PI 3-kinase inhibition of $p21^{ras} \cdot GTP$ loading in 3T3-L1 fibroblasts, CHO-IR cells, or HIRc cells (Fig. 4).

Insulin stimulates $p21^{ras} \cdot GTP$ loading by the increasing guanine nucleotide exchange activity of Sos (12, 36). Return of

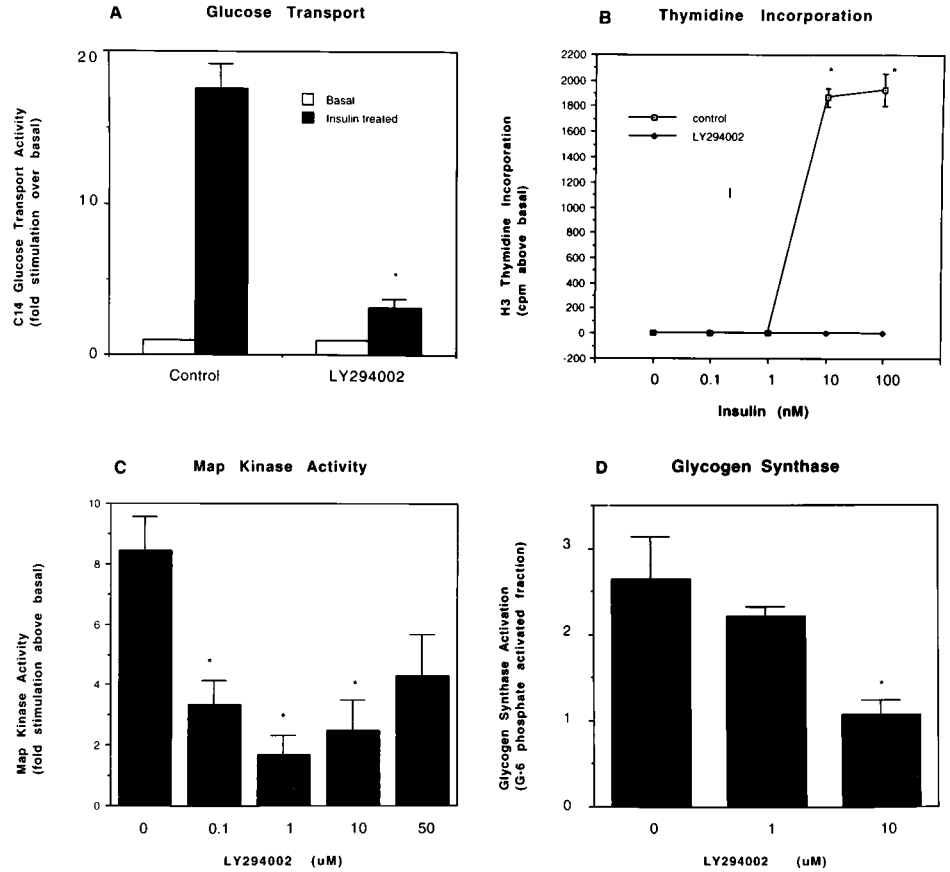

FIG. 1. Effects of the PI 3-kinase inhibitor LY294002 on insulin action in 3T3-L1 adipocytes. (A) Glucose transport activity was assessed in control (basal) and insulin-treated confluent 3T3-L1 cells pretreated with (control) or without LY294002 (10 μM for 5 min). Insulin-stimulated transport activity was 17.6-fold ± 1.6-fold in control cells. This stimulation was significantly blunted (3.1-fold ± 0.6-fold, $P < 0.01$, $n = 4$) in the LY294002-pretreated cells. There was no change in basal transport activity in the two treatment situations. (B) The dose response to insulin-mediated thymidine incorporation was assessed in control and LY294002-pretreated cells as described in Materials and Methods ($n = 4$). Control cells demonstrated a dose-dependent increase in thymidine incorporation in response to insulin which was ablated in the LY294002-pretreated cells ($P < 0.01$). (C) Dose-dependent insulin-stimulated MAP kinase activity toward EGRR peptide in 3T3-L1 adipocytes pretreated with or without LY294002 as described in Materials and Methods ($n = 6$). At doses of 1 μM and higher, LY294002 pretreatment significantly ($P < 0.05$) decreased insulin stimulation of MAP kinase activity. (D) Glycogen synthase activation in response to insulin in control and LY294002-treated cells. LY294002 at 10 μM significantly ($P < 0.05$, $n = 4$) impaired glycogen synthase activation, whereas a dose of 1 μM yielded results was not statistically different from those for control cells.

p21^{ras} into its inactive conformation is facilitated by GAP that hydrolyzes GTP to GDP. Therefore, inhibition of PI 3-kinase activity can impair formation of p21^{ras} · GTP either by inhibiting the guanine nucleotide exchange activity of Sos or by enhancing the activity of GAP. Our previous studies with immunodepletion of PI 3-kinase from lysates of insulin-stimulated cells indicated that the effect of insulin on the guanine nucleotide exchange activity of Sos is not influenced by PI 3-kinase (27). Therefore, in this study, we examined the effect of insulin on GAP activity in 3T3-L1 adipocytes pretreated with the PI 3-kinase inhibitors. GAP activities expressed as percentages of hydrolyzed GAP were 22.9 ± 8.7, 21.7 ± 7.5, 41.8 ± 10, and 67.0 ± 2.5 (means ± SEM of three independent experiments; for the latter two values, $P < 0.01$ versus the corresponding control) for 3T3-L1 adipocytes given no pretreatment, treated without wortmannin and with insulin, treated with wortmannin and without insulin, and treated with both wortmannin and insulin, respectively. In contrast to control cells (in which there was no effect of insulin on GAP activity), inhibition of PI 3-kinase by either LY294002 (Fig. 5) or wortmannin (see above) resulted in significant increases in basal and insulin-stimulated GAP activity, suggesting that this enhanced GAP activity interferes with insulin's ability to stim-

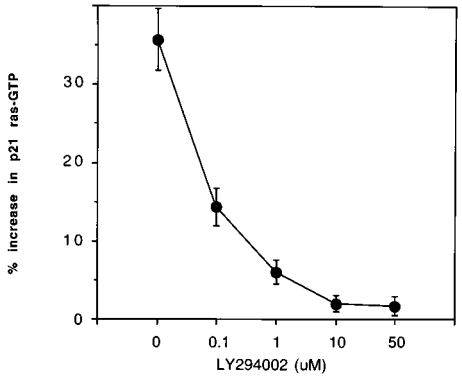

FIG. 2. Effect of LY294002 on activation of p21^{ras} by insulin. 3T3-L1 adipocytes were incubated with LY294002 (0 to 50 μM) for 30 min prior to exposure to insulin (100 nM) for 10 min. LY294002 significantly blocked insulin-stimulated increases in p21^{ras} · GTP formation in a dose-dependent manner. Results are expressed in increments above the basal levels and represent the means ± SEM of three experiments. The basal percentage of p21^{ras} · GTP ranged between 13 and 28.

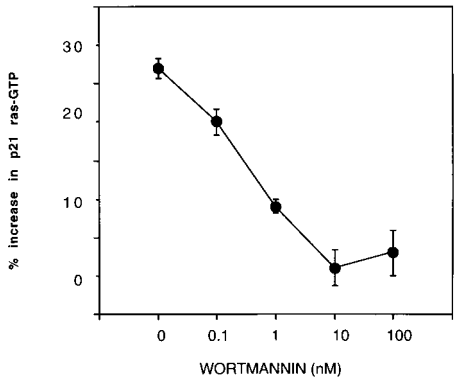

FIG. 3. Effect of wortmannin on activation of p21^{ras} by insulin. 3T3-L1 adipocytes were incubated with wortmannin (0 to 100 nM) for 30 min prior to treatment with insulin (100 nM) for 10 min. Wortmannin significantly blocked insulin-stimulated increases in p21^{ras} · GTP formation in a dose-dependent manner. Results are expressed as described for Fig. 2 and represent the means ± SEM of two experiments.

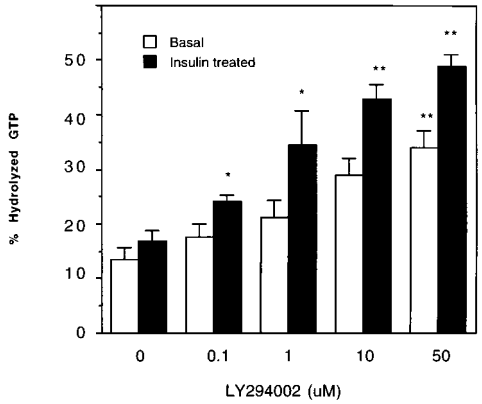

FIG. 5. Effect of LY294002 on GAP activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with LY294002 (0 to 50 μM) for 30 min prior to treatment with insulin (100 nM) for 10 min. LY294002 significantly increased both basal and insulin-stimulated GAP activities in a dose-dependent manner (*, $P < 0.05$; **, $P < 0.01$ versus GAP activity in cells not exposed to LY294002).

ulate p21^{ras} · GTP loading and that in intact cells, PI 3-kinase exerts an important inhibitory effect on GAP.

The mechanism of interaction between the PI 3-kinase and GAP is unknown. Two structurally dissimilar inhibitors of the PI 3-kinase, wortmannin and LY294002, bind directly to the catalytic p110 subunit of the kinase and thereby reduce its activity (41, 66). At the same time, GAP has been shown to coimmunoprecipitate with p85, a regulatory noncatalytic domain of PI 3-kinase (58), suggesting that physical association between p85 and GAP may be important in modulating the GAP activity. To examine the mechanism of interaction between PI 3-kinase and GAP, we attempted to detect GAP in the immunoprecipitates of either p85 or p110. Only p85 precipitates contained GAP and only after stimulation with insulin (Fig. 6). Conversely, immunoprecipitations of GAP detected its association with p85 and not with p110 (Fig. 7). It appears that 3T3-L1 adipocytes possess p85 in excess of p110. p85 antibody immunoprecipitates approximately 90% of cellular p85, whereas p110 antibody appears to completely deplete the lysates of p110 (Fig. 8). Immunoprecipitation of p85 completely depletes the lysates of p110, while complete immunoprecipitation of p110 removes only 80 to 90% of p85 (Fig. 9). Therefore, GAP appears to bind only to p85 not associated with p110. On the other hand, immunodepletion of cell lysates of either p85 or p110 prior to stimulation of these lysates with insulin resulted in an increased basal GAP activity (Fig. 10). These results indicate that removal of the catalytic activity of the p110 subunit of PI 3-kinase (by immunodepletion or by biochemical inhibition) eliminates an inhibitory influence of PI 3-kinase on GAP. Physical association of p85 with GAP does not affect GAP activity, but immunodepletion of p85 with a resultant decrease in the catalytic activity of p110 also disinhibits GAP.

DISCUSSION

PI 3-kinase is a lipid kinase that phosphorylates the D-3 position of the inositol ring of PI, resulting in increased production of the lipid products PI-3-monophosphate, PI-3,4-bisphosphate, and PI-3,4,5-trisphosphate (1, 31, 62). Structurally, PI 3-kinase is a heterodimer composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (43). p85 contains two SH2 domains and one SH3 domain (42, 55). The SH2 domains of p85 bind to the phosphorylated YXXM motifs present in the cytoplasmic domains

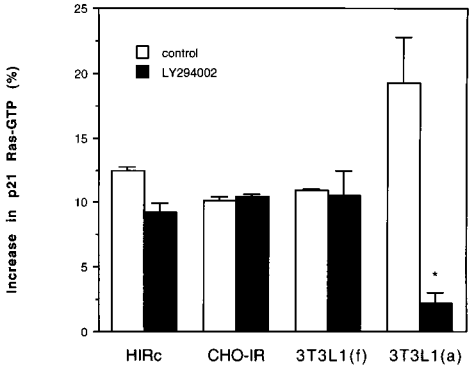

FIG. 4. Effect of LY294002 on insulin-induced p21^{ras} activation in HIRc fibroblasts, CHO-IR cells, 3T3-L1 fibroblasts (f), and 3T3-L1 adipocytes (a). Preincubation of cells with LY294002 for 30 min (50 μM) significantly blocked the increase in p21^{ras} · GTP formation only in 3T3-L1 adipocytes. The increase in the percentage of p21^{ras} · GTP was determined as described in Materials and Methods. Results represent the means ± SEM of two experiments performed in duplicate (*, $P < 0.01$ versus control).

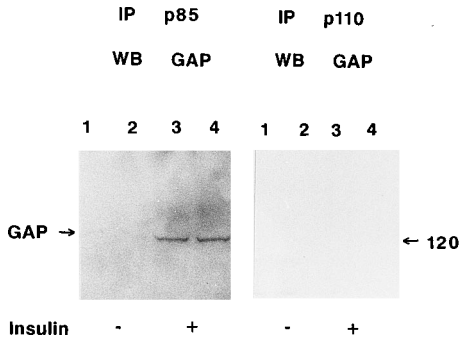

FIG. 6. Coimmunoprecipitation of GAP with p85. 3T3-L1 adipocytes were incubated with (lanes 3 and 4) and without (lanes 1 and 2) insulin (100 nM for 10 min), and lysates were immunoprecipitated (IP) with either p85 or p110 antibody as described in Materials and Methods. Immunoprecipitates were subjected to SDS-PAGE (7.5% polyacrylamide gel) and Western blotted (WB) for GAP. GAP (124 kDa) coimmunoprecipitated with p85, but not with the p110 subunit of PI 3-kinase, and only after insulin treatment. Size on the right is indicated in kilodaltons.

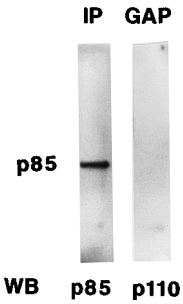

FIG. 7. Coimmunoprecipitation of p85 with GAP. The cells were incubated with insulin (100 nM for 10 min), and lysates were immunoprecipitated (IP) with GAP antibody. Immunoprecipitates were subjected to 7.5% SDS-PAGE (7.5% polyacrylamide gel) and Western blotted (WB) for p85 or p110. Only p85 coimmunoprecipitated with GAP.

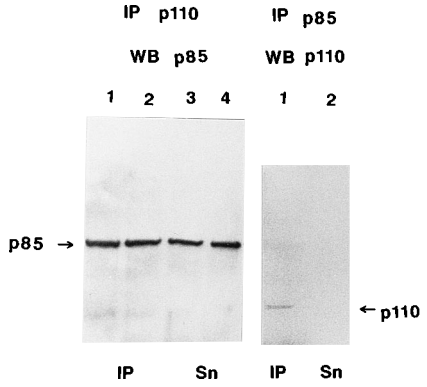

FIG. 9. Coimmunoprecipitations of p110 and p85 in 3T3-L1 adipocytes. Cell lysates were immunoprecipitated (IP) with either p110 or p85 antibody as described in Materials and Methods. Immunoprecipitates (IP) and supernatants (Sn) were analyzed by SDS-PAGE and Western blotted (WB) with either p110 or p85 antibody. Immunoprecipitations of p85 completely depleted the cell lysates of p110. In contrast, immunoprecipitations of p110 did not deplete the lysates of p85, despite complete removal of p110 (Fig. 8).

of cell surface receptors and in IRS-1 (13, 56, 57, 60). This binding event results in conformational changes in the p85 (regulatory subunit) and thereby an activation of the catalytic subunit p110 (50). Other mechanisms of activation of PI 3-kinase include recently described association of Src family SH3 domains to p85 (45) and the binding of $p21^{ras}$ to p110 (49).

Functionally, PI 3-kinase has been shown to participate in mediating the mitogenic effects of various growth factors (6, 8, 10, 24, 56) and insulin-stimulated glucose uptake and membrane ruffling (6, 18, 19, 25, 41, 67). The former effect appears to involve activation of $p70^{S6}$ kinase (6, 8), while the latter was important for the translocation of GLUT-4 to the plasma membrane (6, 37, 41).

Despite recent advances in understanding both the function and the structure of PI 3-kinase, the precise mechanism of its involvement in mediating insulin signaling is not well understood. First, most studies were performed in cells and tissues that lack the full gamut of insulin responsiveness. Tissue-specific interactions among signaling intermediates are now well recognized and must be taken into consideration for meaningful assessment of the mechanism of cellular response to insulin. Second, a great deal of information about the cellular role of PI 3-kinase has been obtained in experiments with growth factors other than insulin, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). These findings may or may not be applicable to insulin, and extrapolations from the experiments with different growth factors into the mechanism of insulin signaling may not be justified. These points were well illustrated by a recent publication of Wiese and colleagues (63), who clearly demonstrated that activation of MAP kinase and PI 3-kinase by EGF or PDGF was not sufficient for the complete stimulation of glucose transport, lipid synthesis, or glycogen synthesis in 3T3-L1 adipocytes. Insulin not only activated PI 3-kinase and MAP kinase, similarly to EGF and PDGF, but unlike these two growth factors, it also stimulated glucose uptake, glycogen, and lipid synthesis (63). These experiments suggest a requirement for other signaling mechanisms that are likely to be uniquely responsive to insulin (63).

These considerations prompted us to investigate the role of PI 3-kinase in mediating insulin signaling in 3T3-L1 adipocytes by using two approaches: (i) inhibition of PI 3-kinase activity by the specific inhibitors LY29004 and wortmannin and (ii) immunodepletion of PI 3-kinase subunits from cell lysates. Predictably and in agreement with previous findings, inhibition of PI 3-kinase activity resulted in abrogation of the insulin effects on glucose uptake and thymidine incorporation (6, 19).

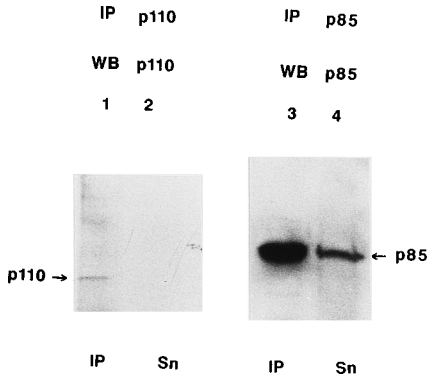

FIG. 8. Immunodepletions of the cell lysates with p110 and p85. The cell lysates were incubated with either p110 and p85 antibody as described in Materials and Methods. Both immunoprecipitates (IP) and supernatants (Sn) were analyzed by SDS-PAGE and Western blotted (WB) with either p110 or p85 antibody. Immunodepletion of p110 was complete, while p85 antibody removed approximately 90% of the cellular p85.

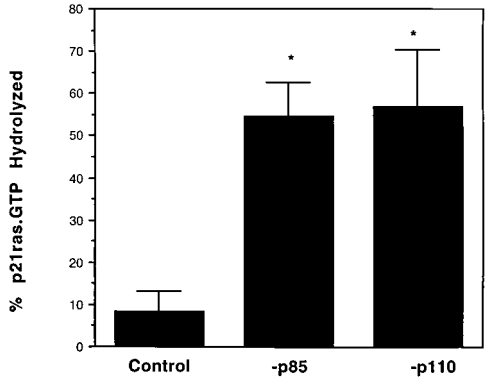

FIG. 10. Effect of immunodepletion of p85 or p110 on GAP activity in the lysates of control and insulin-treated 3T3-L1 adipocytes. The lysates were immunoprecipitated with p85 or p110 antibody as described in Materials and Methods. Postimmunoprecipitation lysates were used for determination of GAP activity. GAP activity was significantly increased (*, $P < 0.05$ versus control, $n = 4$) following immunodepletion of either p85 or p110.

In contrast, however, we found that inhibition of PI 3-kinase resulted in reductions in the fold stimulation of MAP kinase and glycogen synthase activities by insulin. We attribute these differences to the tissue specificity of the response to PI 3-kinase inhibitors. Previously, inhibition of MAP kinase activity in response to wortmannin has been shown but required relatively high concentrations of the PI 3-kinase inhibitor, at least 10- to 50-fold higher than those inhibiting glucose uptake (16).

The unexpected findings included influence of PI 3-kinase on GAP activity and $p21^{ras} \cdot GTP$ loading. This influence appears to be tissue specific, with only differentiated adipocytes being sensitive to PI 3-kinase inhibitors (Fig. 4). Conceivably, adipocytes, representing major insulin-responsive tissue, express unique isoforms of either PI 3-kinase or GAP allowing this particular type of interaction. Whether other insulin-sensitive tissues behave similarly to adipocytes is unknown. It is clear, however, that tissue specificity of signaling interactions must be taken into consideration in assessing the mechanism of insulin action.

Interactions between PI 3-kinase and $p21^{ras}$ appears to be complex. Initial studies suggested almost complete separation of the two pathways, since the inhibition of PI 3-kinase activity did not affect activation of $p21^{ras}$. Recently, however, it was suggested that $p21^{ras} \cdot GTP$ can activate PI 3-kinase (49), providing the first firm indication of cross talk between these signaling intermediates. The most recent findings that several cellular responses to constitutively active PI 3-kinase are Ras dependent (22) strongly indicate the existence of important interactions between the functions of PI 3-kinase and $p21^{ras}$. Our present data not only support this notion but identify at least one mechanism of their interactions.

It appears that in adipocytes, PI 3-kinase prevents activation of GAP. When PI 3-kinase activity was removed by immunodepletion (Fig. 9) or inhibited by LY29004 (Fig. 3) or wortmannin (see Results), GAP activity increased, converting $p21^{ras} \cdot GTP$ into $p21^{ras} \cdot GDP$.

It also appears that preserved catalytic activity of p110 is essential for this interaction. Both inhibitors of PI 3-kinase (wortmannin and LY29004) interfere with p110's catalytic activity. Moreover, immunodepletion of p110 from the cell lysates resulted in increased GAP activity (Fig. 9). Previous studies have shown that GAP associates with p85 (presumably via p62, a GAP-associated protein) (55, 58). This interaction of GAP with p85 has been suggested to modulate GAP activity. Our data suggest that the p85 subunit of the PI 3-kinase does indeed bind GAP, but this association does not influence GAP activity for as long as normal activation of p110 is observed. When p85 is removed by immunodepletion, insulin no longer activates p110 and GAP activity increases significantly.

Original observations have suggested that p85 binds to the cytosolic domains of the tyrosine kinase-containing receptors or to IRS-1 and activates the p110 catalytic subunit of the PI 3-kinase. Newer findings indicate that p85 binds other cytosolic proteins and thus may divert the signal to additional pathways. It appears that p85 belongs to a distinct class of SH2 domain-containing proteins, collectively called adaptor proteins, that include Grb-2 (35), Shc (14), $p47^{gag\text{-}crk}$ (30), and Nck (32). These proteins consist predominantly of SH2 and SH3 domains and are assumed to couple activated tyrosine kinases to various effector pathways (44). In the case of p85, it binds to insulin receptor, IRS-1, p110, and p62-GAP-associated protein (28, 34, 53, 58). The latter has been shown to undergo phosphorylation in response to insulin, v-src, or protein kinase C (PKC) (40, 58, 64) and also to bind phospholipase Cγ (PLCγ) (33, 48). An association of p62 with PLCγ can tie insulin signaling to PI 3-kinase with the PLC- and PKC-dependent system (39). Both PKC and arachidonic acid-related phospholipids have been shown by several investigators to be potent inhibitors of GAP (15, 17, 46, 59, 68). Furthermore, PKC has been shown to activate MAP kinase by both $p21^{ras}$-dependent and -independent mechanisms (3, 4, 9, 21, 26, 29), in a continuous cross talk with the insulin signaling network. Recent findings that PI 3-kinase may stimulate PKC (39) are supportive of this cross talk. Thus, one can envision that PI 3-kinase inhibits GAP via activation of PKC- and diacylglycerol-dependent steps, but this possibility requires experimental confirmation.

In summary, these observations indicate that PI 3-kinase prevents stimulation of GAP, thereby changing the equilibrium in favor of Sos, which promotes $p21^{ras} \cdot GTP$ loading in response to insulin. This effect of PI 3-kinase is dependent upon normal catalytic activity of p110. The role of p85 binding to p62-GAP-associated protein and possible extension of the insulin signal to PLCγ, PKC, and phospholipases remain to be investigated.

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