MOLECULAR AND CELLULAR BIOLOGY, July 1994, p. 4902–4911
0270-7306/94/$04.00+0

Vol. 14, No. 7

Phosphatidylinositol 3-Kinase Activation Is Required for Insulin Stimulation of pp70 S6 Kinase, DNA Synthesis, and Glucose Transporter Translocation

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Received 15 March 1994/Returned for modification 13 April 1994/Accepted 25 April 1994

Phosphatidylinositol 3-kinase (PI 3-kinase) is stimulated by insulin and a variety of growth factors, but its exact role in signal transduction remains unclear. We have used a novel, highly specific inhibitor of PI 3-kinase to dissect the role of this enzyme in insulin action. Treatment of intact 3T3-L1 adipocytes with LY294002 produced a dose-dependent inhibition of insulin-stimulated PI 3-kinase (50% inhibitory concentration, 6 μM) with >95% reduction in the levels of phosphatidylinositol-3,4,5-trisphosphate without changes in the levels of phosphatidylinositol-4-monophosphate or its derivatives. In parallel, there was a complete inhibition of insulin-stimulated phosphorylation and activation of pp70 S6 kinase. Inhibition of PI 3-kinase also effectively blocked insulin- and serum-stimulated DNA synthesis and insulin-stimulated glucose uptake by inhibiting translocation of GLUT 4 glucose transporters to the plasma membrane. By contrast, LY294002 had no effect on insulin stimulation of mitogen-activated protein kinase or pp90 S6 kinase. Thus, activation of PI 3-kinase plays a critical role in mammalian cells and is required for activation of pp70 S6 kinase and DNA synthesis and certain forms of intracellular vesicular trafficking but not mitogen-activated protein kinase or pp90 S6 kinase activation. These data suggest that PI 3-kinase is not only an important component but also a point of divergence in the insulin signaling network.

Insulin-mediated signals regulate a variety of intracellular growth and metabolic events including the uptake and disposal of glucose, transport of amino acids, transcription of specific genes, and synthesis of DNA (22, 35). These signals are initiated by insulin binding to and activating its cell surface receptor tyrosine kinase, resulting in autophosphorylation of the receptor on tyrosine residues and rapid phosphorylation of an immediate downstream substrate molecule, insulin receptor substrate 1 (IRS-1) (25, 55). IRS-1 is a high-molecular-weight cytosolic protein which contains 20 potential tyrosine phosphorylation sites and over 40 potential serine/threonine phosphorylation sites and has been shown to be a major substrate for both the insulin and IGF-1 receptors (32, 48). Several of the potential tyrosine phosphorylation sites reside in peptide sequences that are known to associate with proteins containing SH2 domains (48). In its phosphorylated form, IRS-1 has been shown to act as a docking protein that forms a signaling complex with phosphatidylinositol 3-kinase (PI 3-kinase), the phosphotyrosine phosphatase SHPTP2 (Syp), and GRB-2, a molecule which links IRS-1 to the $p21^{ras}$ signaling system (31, 44, 48). Association of IRS-1 with PI 3-kinase and other proteins containing SH2 domains results in their activation, and IRS-1 thus plays a pivotal role in the complex divergent network encompassing insulin's pleiotropic effects (2, 33, 41).

In addition to its interaction with IRS-1, PI 3-kinase directly associates with and is stimulated by a number of activated growth factor receptors and nonreceptor tyrosine kinases, including the platelet-derived growth factor (PDGF) and colony-stimulating factor 1 receptors and the Src-like kinases (12, 24, 43, 52). PI 3-kinase is a heterodimeric enzyme composed of a p85 regulatory subunit and a p110 catalytic subunit. PI 3-kinase phosphorylates phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PI-4-P), and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) on the D-3 position, producing phosphatidylinositol-3-monophosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2), and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3), respectively (56, 57). The exact physiological roles of the D-3-phosphorylated phosphoinositides (PPIs) are unknown. However, since they are not substrates for any known phospholipases, their biological activities may be mediated as intact lipids (39). An increase in the levels of D-3-phosphorylated PPIs occurs following growth factor stimulation in association with an increase in cellular proliferation (34). In addition, these products have been shown to be involved in cytoskeletal alterations by promoting actin polymerization (21). The *Saccharomyces cerevisiae* homolog of the p110 subunit of PI 3-kinase (VPS34) has also been shown to be required for proper protein sorting (37, 45). Thus, it seems likely that this lipid kinase plays diverse roles in the intact cell.

In order to gain a better understanding of the function of this enzyme, Vlahos et al. (54) have recently synthesized a specific inhibitor of PI 3-kinase, LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]. LY294002 behaves as a competitive inhibitor of the ATP binding site specific for PI 3-kinase and abolishes PI 3-kinase activity in vitro and in vivo at low micromolar concentrations but has no inhibitory effect against PI 4-kinase nor a number of intracellular serine/threonine or tyrosine kinases (54; also see Discussion). In the

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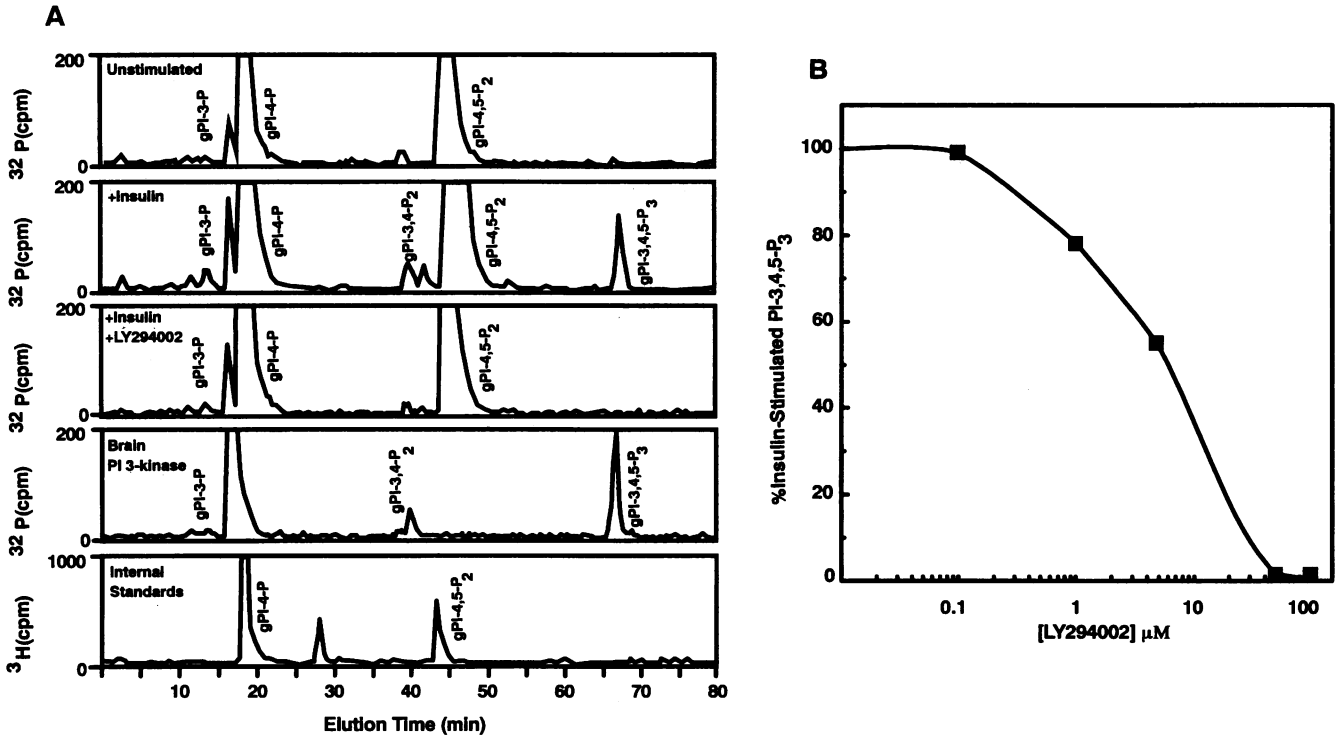

FIG. 1. Inhibition of PI 3-kinase activity in intact 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were serum starved overnight and then radiolabeled for 2 h with $^{32}P_i$. Cells were treated with DMSO (carrier) or 50 μM LY294002 for 10 min and then stimulated with insulin for an additional 10 min. Cells were harvested, and ^{32}P-labeled phospholipids were extracted and separated by TLC. D-3-phosphorylated PPIs were extracted from the TLC plates, deacylated, and analyzed by anion-exchange HPLC as previously described (53). [^{3}H]PI-4-P and [^{3}H]PI-4,5-P_2 were used as internal standards. Retention times were compared with those of [^{32}P]PI-3-P, [^{32}P]PI-3,4-P_2, and [^{32}P]PI-3,4,5-P_3 prepared in vitro with purified bovine brain PI 3-kinase. (B) A dose-response curve for the inhibition of PI-3,4,5-P_3 production by LY294002 was generated by the experimental approach described above. The percent PI-3,4,5-P_3 produced was calculated from the ratio of integrated peak areas of PI-3,4,5-P_3 from insulin-stimulated, LY294002-treated cells compared with that of cells stimulated with insulin alone.

present study we have utilized this specific enzyme inhibitor to investigate the role of PI 3-kinase in insulin-regulated events in 3T3-L1 adipocytes. We find that PI 3-kinase is necessary for glucose transporter translocation and cell growth and is an upstream component in the signaling cascade leading to the phosphorylation and activation of pp70 S6 kinase (pp70^{S6K}) but not pp90 S6 kinase (pp90^{rsk}) or mitogen-activated protein kinase (MAPK).

MATERIALS AND METHODS

Cell culture. 3T3-L1 fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM) containing 2 mM glutamine and 10% newborn calf serum in an atmosphere of 10% CO_2 at 37°C. Differentiation of the fibroblasts to adipocytes was induced by incubating confluent monolayers for 3 to 4 days in DMEM containing 2 mM glutamine, 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), and 0.4 μg of dexamethasone per ml. This medium was removed, and the cells were fed every 3 to 4 days in DMEM containing 2 mM glutamine and 10% fetal bovine serum. Greater than 70% of the cells expressed the adipocyte phenotype by days 10 to 12.

PI 3-kinase activity in intact cells and in vitro. 3T3-L1 adipocytes were serum starved overnight in DMEM containing 0.2% bovine serum albumin (BSA). The cells were then placed in phosphate-free medium containing 0.5 mCi of $^{32}P_i$ (HCl-free; 8,500 to 9,120 Ci/mmol; New England Nuclear) per 10-cm-diameter dish for 2 h at 37°C. LY294002 (final concentration of 0.1 to 100 μM in 160 μl of dimethyl sulfoxide [DMSO]) was added to the plates, which were then incubated an additional 10 min at 37°C. Cells were stimulated with 100 nM insulin for 10 min and then washed twice with ice-cold phosphate-buffered saline. Cells were harvested by scraping them into 750 μl of 1 N HCl-methanol (1:1 [vol/vol]), and phospholipids were extracted as previously described (38). Phospholipids were resuspended in 60 μl of $CHCl_3$ and separated by thin-layer chromatography (TLC) on silica plates treated with 1.2% potassium oxalate. PPIs were extracted from the TLC plates, chemically deacylated with methylamine, and subjected to anion-exchange high-pressure liquid chromatography (HPLC) with an on-line radiochemical detector (53); their retention times were compared with those of deacylated [^{32}P]PI-3-P, [^{32}P]PI-3,4-P_2, and [^{32}P]PI-3,4,5-P_3 produced by using purified bovine PI 3-kinase. Deacylated [^{3}H]PI-4-P and [^{3}H]PI-4,5-P_2 were used as internal standards. Quantitation was determined by measuring integrated counts per minute for the PI-3,4,5-P_3 peak as a percentage of control. In vitro PI 3-kinase activity was determined by phosphorylation of PI with anti-IRS-1 (αIRS-1) or anti-p85 (αp85) immunoprecipitates (3, 4). Reaction products were visualized and quantitated with a Molecular Dynamics PhosphorImager. For immunoblotting of PI 3-kinase, we used a mouse monoclonal αp85 antibody (clone PK4; Transduction Laboratories). The immunoblots were visualized by using anti-mouse immunoglobulin G cou-

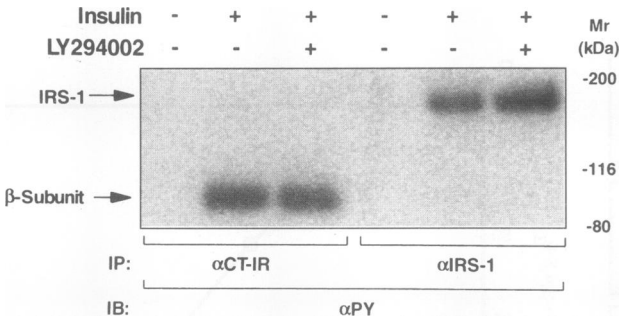

FIG. 2. Effect of LY294002 on insulin receptor and IRS-1 phosphorylation. Serum-starved 3T3-L1 adipocytes were incubated for 5 min in the presence (+) or absence (−) of 50 μM LY294002 and then stimulated with insulin (100 nM) (+) or not stimulated (−). Cell extracts were prepared and subjected to immunoprecipitation (IP) with anti-insulin receptor (αCT-IR) or αIRS-1 antibodies. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted (IB) with an antiphosphotyrosine antibody (αPY).

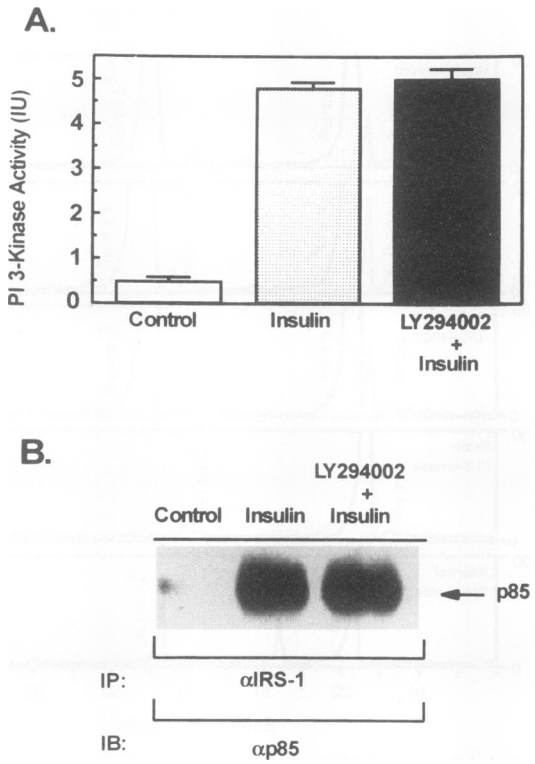

FIG. 3. Effect of LY294002 on the association of PI 3-kinase with IRS-1 in intact cells. (A) Adipocytes were incubated for 5 min with DMSO (control) or LY294002 (50 μM) and treated with insulin (100 nM) for 1 min or not treated with insulin. PI 3-kinase activity was determined on αIRS-1 immunoprecipitates in triplicate and quantitated as described in Materials and Methods. Data are presented in arbitrary PhosphoImager units (IU). (B) The αIRS-1 immunoprecipitates from the cell lysates described above were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting with a monoclonal αp85 antibody (Transduction Laboratories). IP, immunoprecipitation; IB, immunoblotting.

pled to horseradish peroxidase and the enhanced chemiluminescence (Amersham) detection kit.

Insulin-stimulated phosphorylation. Serum-starved adipocytes were treated with DMSO (control) or LY294002 (50 μM) for 5 min at 37°C, followed by stimulation with 100 nM insulin for 1 min. Cell extracts were prepared (8) and incubated with a polyclonal antibody raised against the C-terminal 15 amino acids of the human insulin receptor (αCT-IR) or αIRS-1 and precipitated with protein A-Sepharose (Pierce). The immunoprecipitated proteins were subjected to immunoblotting with a polyclonal antiphosphotyrosine antibody (αPY) and then with ^{125}I-labeled protein A and visualized by autoradiography or by using a Molecular Dynamics PhosphorImager.

DNA synthesis. For serum- and insulin-stimulated DNA synthesis, 3T3-L1 fibroblasts were grown in 12-well cluster trays and serum starved for 2 days in DMEM containing 0.1% BSA. The cells were incubated with or without LY294002 in the presence or absence of insulin (1 μM) or calf serum (10%) for 15 h and then incubated with [^{3}H]thymidine (1 μCi per well) for 1 h. Trichloroacetic acid-precipitable material was collected on glass-fiber filters and washed, and the radioactivity was determined by scintillation counting.

Glucose uptake and glucose transporter translocation. 3T3-L1 fibroblasts were grown to confluency in 12-well dishes and differentiated as described above. The adipocytes were washed twice in KRP transport buffer (137 mM NaCl, 4.7 mM KCl, 10 mM sodium phosphate [pH 7.4], 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% BSA), serum starved for 2 h in DMEM containing 0.2% BSA, and washed twice in KRP transport buffer, and a glucose-free incubation was done in transport buffer for 45 min (14). Cells were incubated with LY294002 and then treated with 100 nM insulin for 15 min. During the last 4 min of insulin stimulation, glucose uptake was initiated by the addition of 2-[1,2-^{3}H]deoxy-D-glucose (0.5 μCi/ml; 30 Ci/mmol) and 100 μM unlabeled 2-deoxy-D-glucose. Nonspecific uptake was determined in the presence of 10 μM cytochalasin B.

GLUT 4 glucose transporter translocation was performed as previously described with plasma membrane sheets (14, 16). Cells were grown and differentiated on coverslips and treated with insulin and LY294002 as described above for glucose uptake. Plasma membrane sheets were prepared by sonification, and GLUT 4 present in the membranes was visualized by immunofluorescence with a polyclonal anti-GLUT 4 antisera (kindly provided by Diane C. Fingar and Morris J. Birnbaum, Harvard Medical School) and a rhodamine-conjugated secondary antibody.

MAPK, pp70^{S6K}, and pp90^{rsk} assays. Cell lysates were prepared as described above for phosphorylation except for the addition of 0.2% Brij 35. Immunoprecipitations and protein kinase assays were performed by using 40S ribosomal subunit S6 as the substrate for pp70^{S6K} and pp90^{rsk} and recombinant Rsk as a substrate for MAPK (9). Rabbit polyclonal antibodies for pp70^{S6K} were generated against an N-terminal peptide (amino acids 20 to 39), and MAPK antibodies were raised against the C-terminal 20 amino acids. Antibodies against pp90^{rsk} were raised against the full-length chicken Rsk protein (6).

RESULTS

Effect of LY294002 on PI 3-kinase in 3T3-L1 adipocytes. 3T3-L1 adipocytes were labeled with $^{32}P_i$, incubated for 10 min in the presence or absence of LY294002, and then stimulated with insulin (100 nM) for 10 min. HPLC analysis of deacylated PPIs demonstrated an insulin-stimulated increase in the production of PI-3,4-P_2 and PI-3,4,5-P_3 (Fig. 1A, +Insulin panel). LY294002 was effective in inhibiting both basal and insulin-

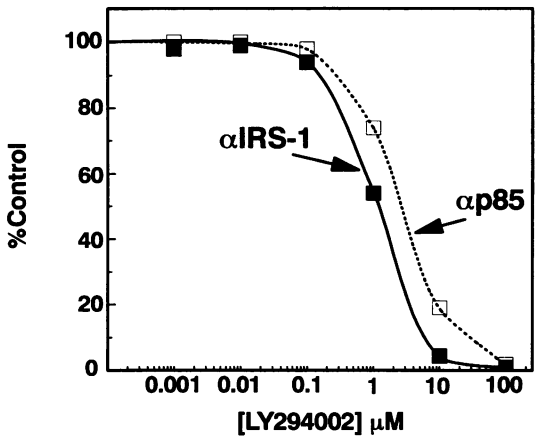

FIG. 4. Inhibition of PI 3-kinase associated with immunoprecipitates of IRS-1 or p85. Lysates from insulin-stimulated 3T3-L1 adipocytes were immunoprecipitated with αIRS-1 or αp85 and protein A-Sepharose. The immunocomplexes were washed and incubated for 5 min with various concentrations of LY294002 (0 to 100 μM), and PI 3-kinase activity was determined. The reaction products were visualized and quantitated with a Molecular Dynamics PhosphoImager. The data are presented as percent control (DMSO alone), and each point represents the average from two independent experiments where each point was determined in triplicate.

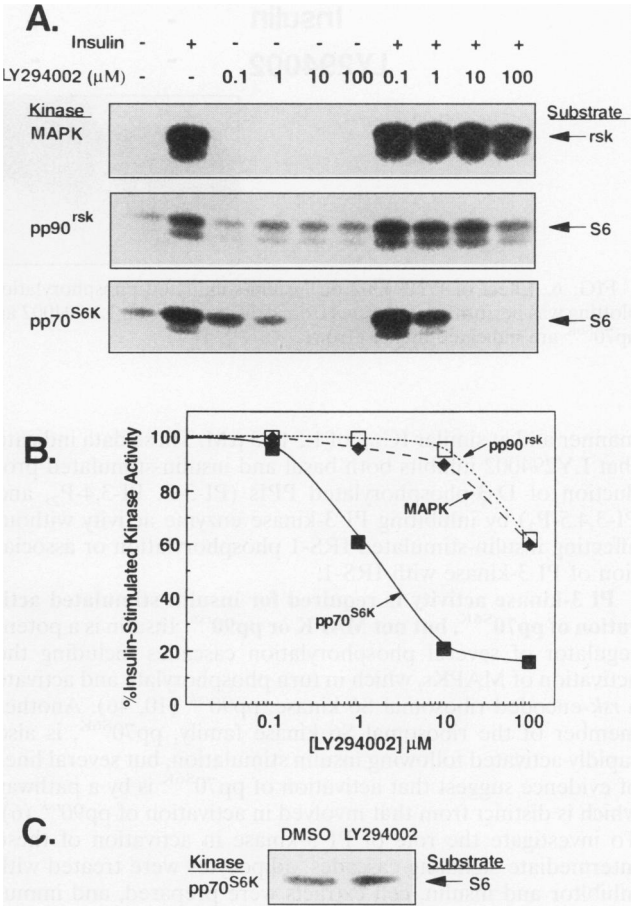

FIG. 5. Effect of LY294002 on activation of MAPK, pp90^{rsk} and pp70^{S6K}. (A) 3T3-L1 adipocytes were serum starved overnight, incubated in the presence or absence (−) of the indicated concentrations of LY294002 for 5 min, and then stimulated with insulin (+) for an additional 5 min or not stimulated (−). Cell lysates were prepared, and immunocomplex assays for MAPK, pp90^{rsk} and pp70^{S6K} were performed as described in Materials and Methods. 40S ribosomal subunits were used as the substrate in the pp90^{rsk} and pp70^{S6K} assays, with the bands appearing in these lanes being phosphorylated S6. Recombinant Rsk was used as the substrate in MAPK assays. The phosphorylated products from these reactions were separated by SDS-polyacrylamide gel electrophoresis, visualized, and quantitated with a Molecular Dynamics PhosphorImager. The data presented are representative of multiple experiments with identical results. (B) Data from the experiment in panel A were plotted as a percentage of the insulin-stimulated MAPK, pp90^{rsk}, or pp70^{S6K} activity (as indicated) versus increasing concentrations of LY294002. (C) Immunocomplexed pp70^{S6K} was prepared from insulin-stimulated adipocytes and incubated with DMSO or 50 μM LY294002 for 5 min prior to the kinase assay.

stimulated PI 3-kinase activity with more than a 95% decrease in the levels of PI-3,4-P_2 and PI-3,4,5-P_3 but with no effect on the levels of PI-4-P and PI-4,5-P_2 (Fig. 1A, +Insulin +LY294002 panel). Quantitation of the PI-3,4,5-P_3 produced in cells treated with 0.1 to 100 μM LY294002 yielded a 50% inhibitory concentration (IC_{50}) of 6 μM (Fig. 1B), which is similar to the IC_{50} for LY294002 against purified PI 3-kinase in a cell-free system (reference 54 and data presented below).

Effects of LY294002 on insulin receptor and IRS-1 phosphorylation and association of PI 3-kinase with IRS-1. Insulin stimulation of PI 3-kinase activity is dependent on insulin stimulation of receptor kinase activity, IRS-1 phosphorylation, and binding of the phosphorylated IRS-1 to the p85 subunit of PI 3-kinase with activation of the catalytic activity in the p110 subunit (2). To determine if the effect of LY294002 on insulin-stimulated PI 3-kinase activation was due to alterations in either insulin-stimulated receptor phosphorylation and/or phosphotransferase activity, 3T3-L1 adipocytes were incubated in the presence or absence of 50 μM LY294002 and analyzed for tyrosine phosphorylation by immunoblotting of cell extracts with antiphosphotyrosine (αPY) antibodies before and after insulin stimulation. As previously described (25, 55), insulin stimulated the rapid tyrosine phosphorylation of the insulin receptor and IRS-1 (Fig. 2). Preincubation of the cells with LY294002 had no effect either on insulin receptor autophosphorylation or on the receptor's ability to phosphorylate IRS-1. Furthermore, when αIRS-1 immunoprecipitates from adipocytes which had been treated with or without 50 μM LY294002 were assayed for PI 3-kinase activity, insulin stimulated a 10-fold increase in PI 3-kinase activity associated with αIRS-1 immunoprecipitates (Fig. 3A). This is not unexpected, since LY294002 is a reversible inhibitor of PI 3-kinase (54), and no compound would be expected to be present in assays conducted following the extensive washing of the immunoprecipitates. In addition, similar amounts of p85 protein were associated with IRS-1 following treatment with insulin, as determined by immunoblotting the αIRS-1 immunoprecipitates with a mouse monoclonal αp85 antibody, regardless of whether or not the cells were treated with LY294002 (Fig. 3B).

To assess the effect of the inhibitor directly on PI 3-kinase activity associated with IRS-1, cell extracts were prepared from insulin-stimulated 3T3-L1 adipocytes and subjected to immunoprecipitation with either αp85 or αIRS-1. After the immunoprecipitates were washed, they were incubated in the absence or presence of increasing concentrations of LY294002 and PI 3-kinase activity was determined. As shown in Fig. 4, inhibition of PI 3-kinase activity associated with either αp85 or αIRS-1 immunoprecipitates occurred in a dose-dependent

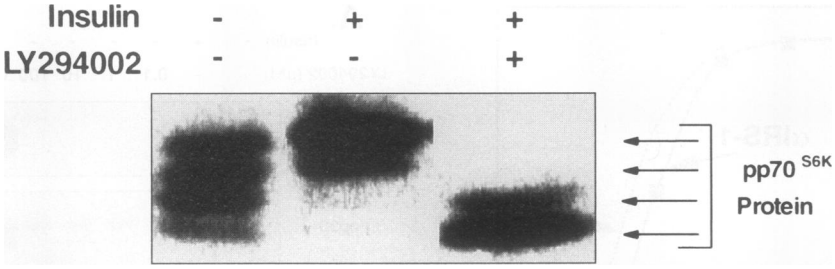

FIG. 6. Effect of LY294002 on insulin-stimulated phosphorylation of pp70^{S6K}. To examine the phosphorylation state of pp70^{S6K}, immunoblotting was performed on lysates from cells treated with LY294002 and/or insulin as indicated above the gel. The various forms of phosphorylated pp70^{S6K} are indicated by the arrows.

manner and at similar IC_{50}s of 1.5 to 3 μM. These data indicate that LY294002 inhibits both basal and insulin-stimulated production of D-3-phosphorylated PPIs (PI-3-P, PI-3,4-P_2, and PI-3,4,5-P_3) by inhibiting PI 3-kinase enzyme activity without affecting insulin-stimulated IRS-1 phosphorylation or association of PI 3-kinase with IRS-1.

PI 3-kinase activity is required for insulin-stimulated activation of pp70^{S6K}, but not MAPK or pp90^{rsk}. Insulin is a potent regulator of several phosphorylation cascades including the activation of MAPKs, which in turn phosphorylate and activate a *rsk*-encoded ribosomal S6 kinase, pp90^{rsk} (10, 46). Another member of the ribosomal S6 kinase family, pp70^{S6K}, is also rapidly activated following insulin stimulation, but several lines of evidence suggest that activation of pp70^{S6K} is by a pathway which is distinct from that involved in activation of pp90^{rsk} (6). To investigate the role of PI 3-kinase in activation of these intermediate signaling cascades, adipocytes were treated with inhibitor and insulin, cell extracts were prepared, and immunocomplex assays were carried out as described above with 40S ribosomes as the substrate for pp90^{rsk} and pp70^{S6K} and with recombinant Rsk as the substrate for MAPK (9). Insulin stimulated the activation of MAPK by 10-fold, the activation of pp90^{rsk} by 3-fold, and the activation of pp70^{S6K} by 5-fold (Fig. 5A). When cells were pretreated with LY294002, both basal and insulin-stimulated activation of pp70^{S6K} was dramatically inhibited. This effect was dose dependent with an IC_{50} of 1 to 2.5 μM (Fig. 5B) similar to that seen for inhibition of PI 3-kinase activity. This inhibitory effect was not due to a direct effect of LY294002 on pp70^{S6K}. Indeed, there was no effect of incubation with 50 μM LY294002 on the kinase activity assayed in vitro (Fig. 5C). Furthermore, there was no effect of LY294002 on the activation of either MAPK or pp90^{rsk} at concentrations up to 10 μM and only a slight inhibition of activity at concentrations as high as 100 μM. In vitro activity of these enzymes in the presence of 50 μM LY294002 was also unaffected (data not shown).

In intact cells, stimulation of pp70^{S6K} activity is associated with its phosphorylation on serine and threonine residues, resulting in a reduced mobility of the protein on sodium dodecyl sulfate (SDS)-polyacrylamide gels (6). The upstream components involved in the phosphorylation of pp70^{S6K} are unknown. We investigated the effects of LY294002 on the state of phosphorylation of pp70^{S6K} by immunoblot analysis. pp70^{S6K} detected in lysates from insulin-stimulated cells showed a decrease in the mobility of pp70^{S6K}, indicative of phosphorylation and activation of the protein (Fig. 6). LY294002 blocked the phosphorylation of pp70^{S6K}, as shown by the alteration in the mobility pattern of pp70^{S6K} back to the faster-migrating species. These data suggest that PI 3-kinase is an upstream component required for mediating the phosphorylation and activation of pp70^{S6K}.

PI 3-kinase activity is essential for DNA synthesis. The exact roles of the PI 3-kinase reaction products (PI-3-P, PI-3,4-P_2, and PI-3,4,5-P_3) are unknown, but several lines of evidence implicate them as important for cell growth (1). To determine the effects of inhibiting PI 3-kinase activity on DNA synthesis, 3T3-L1 preadipocytes were serum starved followed by a 12-h incubation in serum-free medium or medium containing 1 μM insulin or 10% calf serum in the presence of 50 μM LY294002. Insulin stimulated an approximately 5-fold increase in thymidine incorporation into DNA compared with a 20-fold increase in cells stimulated with 10% calf serum (Fig. 7). In the presence of LY294002, both insulin- and serum-stimulated effects on DNA synthesis were blocked (IC_{50} of 2 to 5 μM, data not shown). This effect was reversible. If cells that had been pretreated with inhibitor were washed and stimulated with

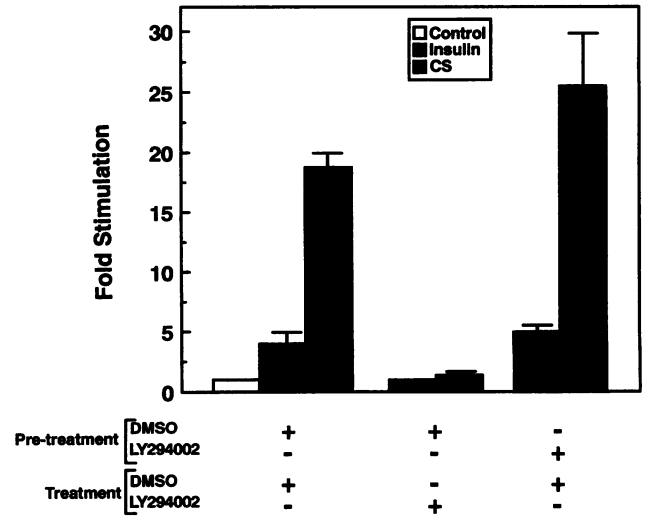

FIG. 7. [^{3}H]Thymidine incorporation in 3T3-L1 fibroblasts following treatment with LY294002. Undifferentiated 3T3-L1 fibroblasts were serum starved for 48 h and pretreated with either DMSO or 50 μM LY294002 for 12 h. The cells were then washed twice in serum-free DMEM, treated with DMSO or 50 μM LY294002, and stimulated with 1 μM insulin or 10% calf serum (CS), and thymidine incorporation was determined after an additional 12 h incubation. The data are presented as fold stimulation above control (treated with DMSO alone) and represent the average (± standard error) from multiple experiments where each point was determined in triplicate.

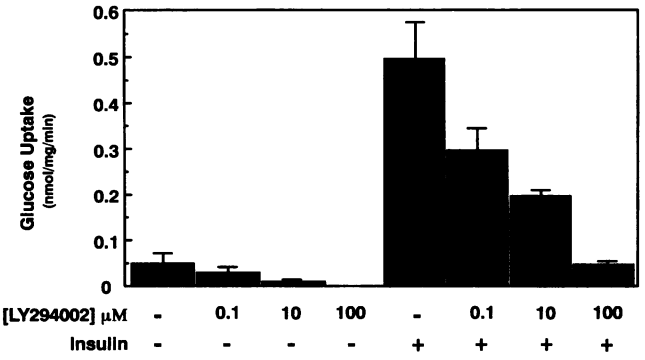

FIG. 8. Inhibition of glucose uptake by LY294002. Fully differentiated 3T3-L1 adipocytes were deprived of serum and glucose and incubated with the indicated concentrations of LY294002 for 5 min, followed by incubation in the presence (+) or absence (−) of insulin (100 nM) for 15 min. Glucose uptake was determined during the last 4 min of insulin treatment by the addition of 100 μM [^{3}H]2-deoxy-D-glucose. Each point was determined in triplicate, and the data presented are representative of three independent experiments.

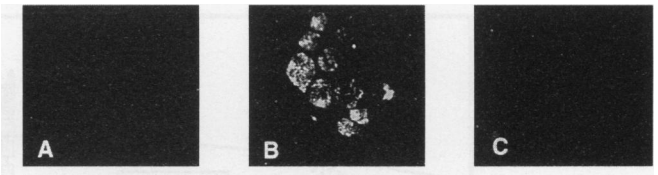

FIG. 9. Effect of LY294002 on GLUT 4 translocation. Plasma membrane sheets were prepared for immunofluorescence from control 3T3-L1 adipocytes (A) or adipocytes stimulated with insulin (B) or treated with 50 μM LY294002 for 5 min followed by insulin as described for glucose uptake (C). The fixed membranes were incubated with anti-GLUT 4 antibody followed by incubation with a rhodamine-conjugated secondary antibody.

agonist, thymidine incorporation was restored to the levels observed in cells that had not been exposed to LY294002 (Fig. 7). Thus, LY294002 inhibits cell growth in a reversible manner and is not cytotoxic. Similar results were observed in Chinese hamster ovary cells expressing the human insulin receptor (data not shown).

Effect of inhibition of PI 3-kinase activity on glucose uptake. The major targets for insulin-stimulated glucose uptake are muscle and adipose tissue (5). 3T3-L1 adipocytes provide an excellent cell culture model for analyzing this insulin-regulated event, since they express the major insulin-sensitive glucose transporter GLUT 4 (14). Incubation of differentiated 3T3-L1 cells with 100 nM insulin for 15 min resulted in a 10-fold increase in glucose uptake (Fig. 8). In contrast, when the cells were preincubated for 10 min in the presence of increasing concentrations of LY294002, both basal and insulin-stimulated glucose transport were blocked with an IC_{50} of 3 to 4 μM, identical to that observed for inhibition of PI 3-kinase. This effect was also seen when LY294002 was added simultaneously with insulin (data not shown). Since the adipocytes need to be exposed to insulin for at least 15 min to achieve a maximal insulin-stimulated glucose uptake, it is difficult to determine how rapidly LY294002 blocks insulin-stimulated glucose uptake, but basal glucose uptake was maximally inhibited within 5 min of exposure to 20 μM LY294002 (data not shown).

In the absence of insulin, GLUT 1 is found predominantly on the cell surface, while GLUT 4 is localized to an intracellular vesicular pool which partitions with a subcellular membrane fraction referred to as the low-density microsomes. Following stimulation with insulin, vesicles associated with the low-density microsome fraction containing GLUT 4, and to a lesser extent GLUT 1, are redistributed to the plasma membrane (13, 20, 59). The mechanism which governs this translocation event is largely unknown. To assess the effects of LY294002 on this insulin-regulated process, GLUT 4 translocation to the plasma membrane was measured by an immunofluorescence assay on plasma membrane sheets prepared from control and insulin-stimulated cells as previously described (14, 16). Compared with control cells, insulin stimulated a significant increase in the amount of GLUT 4 associated with the plasma membrane (Fig. 9), consistent with an insulin-dependent redistribution and fusion of GLUT 4-containing vesicles with the plasma membrane (7, 16). Pretreatment of the adipocytes with LY294002 abolished the insulin-stimulated GLUT 4 translocation (Fig. 9C).

DISCUSSION

PI 3-kinase activity has been found to be associated with a number of protein tyrosine kinases through the binding of its p85 subunit SH2 domains to specific tyrosine phosphopeptide sequences. Association of PI 3-kinase with the insulin signaling pathway occurs through a somewhat different mechanism in that the p85 subunit interacts predominantly with tyrosine phosphorylated IRS-1 rather than the insulin receptor itself (48). In both types of association, the result is activation of the catalytic p110 subunit of PI 3-kinase and a increase in the levels of D-3-phosphorylated PPIs. In this study we have characterized the effects of a specific PI 3-kinase inhibitor on several insulin-regulated cellular events. LY294002 specifically inhibits PI 3-kinase both in vitro and in vivo with an IC_{50} of 1 to 5 μM without having any effect on PI 4-kinase, diacylglycerol kinase, rabbit kidney ATPase, receptor tyrosine kinases (PDGF, epidermal growth factor, and insulin receptor), c-Src kinase, protein kinase A, protein kinase C, MAPK, and S6 kinases (54; also this study). In the present study we find that inhibition of PI 3-kinase results in a parallel blockade of basal and insulin-stimulated glucose uptake, DNA synthesis, and $pp70^{S6K}$ activity but not insulin stimulation of $pp90^{rsk}$ or MAPK activation. These data suggest that D-3-phosphorylated PPIs are necessary for the insulin stimulation of glucose uptake, DNA synthesis, and signaling by $pp70^{S6K}$, as well as, to some extent, maintaining basal levels of activity of these processes.

Wortmannin is a fungal antibiotic which has been shown to be a potent inhibitor of PI 3-kinase and is structurally distinct from LY294002 (58). To address possible concerns about the specificity of LY294002, we analyzed the effects of this alternative PI 3-kinase inhibitor on the insulin-regulated processes in the 3T3-L1 adipocytes. Like LY294002, wortmannin blocked the insulin-stimulated activation of $pp70^{S6K}$ and inhibited glucose uptake (data not shown). Furthermore, wortmannin has been shown to inhibit GLUT 4 translocation in CHO cells transfected with a GLUT 4 cDNA (23). These results provide evidence that PI 3-kinase is required for a number of diverse cellular functions and acts as an upstream component affecting mitogenic potential and intracellular protein trafficking (Fig. 10).

The production of PI-3,4-P_2 and PI-3,4,5-P_3 has been associated with an increase in cell proliferation (34, 50). A decrease in the ligand-dependent production of these signaling molecules and a parallel decrease in mitogenic potential has been observed in cells expressing "kinase insert" mutants of the

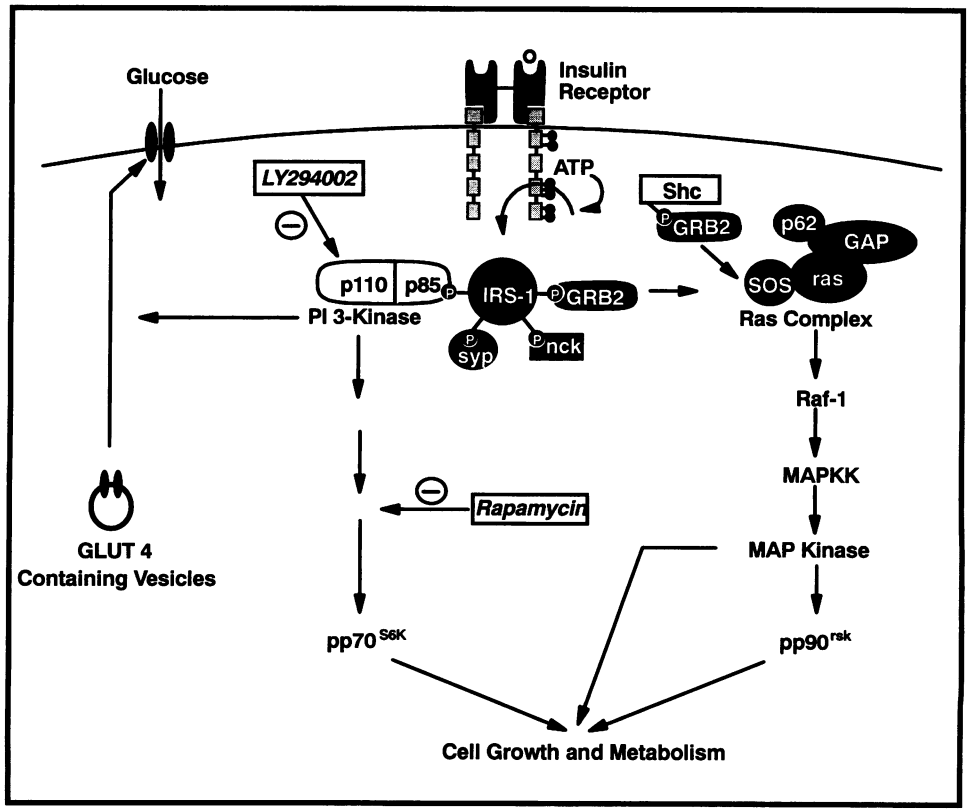

FIG. 10. PI 3-kinase is a diverging point in the insulin signaling network. This model depicts several insulin-regulated pathways and some of the components involved. PI 3-kinase activity is required for glucose transport and activation of pp70^{S6K} but is not involved in regulation of the Ras signaling pathway leading to activation of MAPK and pp90^{rsk}. IRS-1 binds to several proteins containing SH2 domains, including the p85 subunit of PI 3-kinase, the protein-tyrosine phosphatase Syp, and two adaptor molecules, Nck and GRB2. The Ras signaling complex includes the GTPase-activating protein GAP, the p62 GAP-associated protein, and the guanine nucleotide exchange factor SOS. Activation of the Ras signaling complex results in stimulation of a phosphorylation cascade which regulates the activity of several kinases, including the Raf-1 kinase, MAPK kinase (MAPKK), MAPK, and pp90^{rsk}.

PDGF and colony-stimulating factor 1 receptors that have a reduced ability to associate with PI 3-kinase (42, 50). Mutants of polyomavirus middle T antigen and variants of the *abl* oncogene which fail to activate PI 3-kinase are also transformation defective (30, 51). Data presented here demonstrate that insulin also stimulates a dramatic increase in PI-3,4-P_2 and PI-3,4,5-P_3 in 3T3-L1 adipocytes. Inhibition of this PI 3-kinase activity and production of PI-3-P derivatives by LY294002 prevents cells from entering S phase in response to stimulation with serum or insulin.

Our data also strongly suggest that PI 3-kinase is an upstream component in the pathway of insulin stimulation of pp70^{S6K}. In agreement with these findings, it has recently been shown that an in vitro-mutated PDGF receptor that displays a decreased ability to associate with and activate PI 3-kinase also does not activate pp70^{S6K} following treatment with PDGF (10a). These findings are interesting in view of the recent evidence that inhibition of pp70^{S6K} activation by rapamycin delays entry of Swiss 3T3 cells into S phase (11, 28) and that microinjection of antibodies against pp70^{S6K} into rat embryo fibroblasts inhibits serum-stimulated cell cycle progression (29). Thus, the inability of the cells to enter S phase following treatment with LY294002 is due, at least in part, to a loss of pp70^{S6K} function.

Like LY294002, rapamycin blocks activation of pp70^{S6K}, but in contrast to LY294002, rapamycin has no effect on glucose transport in 3T3-L1 adipocytes (16). In view of these data, it seems likely that the inhibition of pp70^{S6K} activation and inhibition of GLUT 4 translocation by LY294002 are separate events requiring unique signals from PI 3-kinase. Thus, while PI 3-kinase activity is required for both the activation of pp70^{S6K} and transport of glucose, the pathway leading to these events appears to branch at some point downstream of PI 3-kinase, and in the case of pp70^{S6K}, upstream of the rapamycin target (Fig. 10). The effect of rapamycin to inhibit pp70^{S6K} activation requires its binding to the FK506-binding protein (FKBP). The target of FKBP-rapamycin in mammalian cells is unknown. TOR2 and DRR1 are yeast homologs of PI 3-kinase that have high sequence similarity to both VPS34 and bovine p110 in regions coding for protein kinase function (27). Although PI 3-kinase activities for the products of TOR2 and DRR1 have not been described, based on genetic evidence, they are candidates for rapamycin targets in *S. cerevisiae*.

Although PI 3-kinase has been most studied in association with protein tyrosine kinases and cell proliferation, there are several lines of evidence which suggest that PI 3-kinase is a component in protein sorting, vesicular transport, and cytoskeletal reorganization. First, when the p110 subunit of bovine PI 3-kinase was cloned, it was found to have striking homology to the VPS34 gene of *S. cerevisiae* (17). Indeed, it has since been shown that the VPS34 gene product is a membrane-associated PI 3-kinase (37). Strains of *S. cerevisiae*

that contain point mutations within the VPS34 gene or that lack the VPS34 gene do not contain any detectable PI 3-kinase activity. Furthermore, these strains are defective in protein sorting, resulting in secretion of precursors of hydrolytic enzymes originally destined for the yeast vacuole (45). Second, in mammalian cells, an increase in PI 3-kinase activity has also been associated with vesicular transport events in actin polymerization (cytoskeletal reorganization) in neutrophils following activation with fMet-Leu-Phe (15, 19, 47, 49). Finally, in rat adipocytes, insulin-stimulated PI 3-kinase activity is found predominantly in the low density microsomes (26), a fraction that is also enriched in Golgi apparatus, secretory vesicles, and GLUT 4 glucose transporters. It is interesting to note that the effects described above occur in nonproliferative cell types, indicating that PI 3-kinase is required for events unrelated to cell growth. Our biochemical and immunofluorescence data showing the effects of LY294002 on glucose transport and GLUT 4 translocation provide evidence that PI 3-kinase is necessary for certain types of vesicle trafficking in mammalian cells.

The mechanisms involved in glucose transport require at least two major steps; one which requires vesicular translocation of glucose transporters from an intracellular membrane fraction and fusion with the plasma membrane and a second endocytotic step again requiring vesicle trafficking and fusion events. Stack et al. (45) have suggested several interesting models that may explain the role of D-3-phosphorylated PPIs in these events. For example, a change in the charge on the head group of a membrane phospholipid might alter local membrane structure, causing a curvature of the bilayer which could initiate a budding or fusion event. Alternatively the presence of D-3-phosphorylated PPIs may serve as markers for recruiting and/or activating membrane components required for protein sorting. In a similar manner, one could envision D-3-phosphorylated PPIs acting as molecular labels that help designate a particular vesicle to a specific trafficking pattern within the cell (36). The ability to discern the potentially separate roles for each of the D-3-phosphorylated PPIs and to define other components involved should provide more insight into this complex cellular function.

Several questions arise from these studies. Are there different roles for PI-3-P, PI-3,4-P_2, and PI-3,4,5-P_3? What are their immediate downstream effector molecules? What are the common properties of such diverse intracellular functions (proliferation versus vesicular transport) that require the activity and products of this unique lipid kinase? Is more than one form of PI 3-kinase in mammalian cells responsible for the diverse functional requirements? Certainly more information is needed to address the first few questions, but both vesicular trafficking and proliferation require cytoskeletal alterations. The latter question has been addressed in part in the yeast system. Both TOR2 and the VPS34 gene products are PI 3-kinase homologs and are necessary for different cellular functions (growth versus vesicular trafficking). In an analogous fashion, this may also be true for mammalian systems. Two separate PI-3-kinase activities have been purified from bovine brain (40). One is the heterodimeric form composed of 85- and 110-kDa subunits (PI3KII), and the other (PI3KI) is a monomeric 110-kDa form. The biochemical properties (specific activity and Ca^{2+} requirements) of these kinases are different, suggesting they may also be physiologically distinct. More recently, the cloning of a second novel and ubiquitously expressed human PI 3-kinase p110 isoform was described, providing evidence for a family of PI 3-kinase genes coding for distinct catalytic subunits (18). Defining the physiological difference between putative PI 3-kinase isoforms and the potential role(s) for each of the D-3-phosphorylated PPIs will clearly provide more information in understanding the mechanism for the regulation of these events.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants CA 46595 (to J.B.) and DK 33201 and DK 31036 (to C.R.K.) and the Joslin DERC (DK 36836) and NIH Training Grant DK07260 from NIDDKD. B.C. is a recipient of a fellowship from The Medical Foundation/Charles A. King Trust, Boston, Mass. J.B. is an established investigator of the American Heart Association.

We also thank Tony Gardner for expert technical assistance.

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