Insulin Regulation of Phosphoenolpyruvate Carboxykinase Gene Expression Does Not Require Activation of the Ras/Mitogen-activated Protein Kinase Signaling Pathway*

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Expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting step in hepatic gluconeogenesis, is primarily regulated at the level of gene transcription. Insulin and phorbol esters inhibit basal PEPCK transcription and antagonize the induction of PEPCK gene expression by glucocorticoids and glucagon (or its second messenger cAMP). Insulin activates a signaling cascade involving Ras → Raf → p42/p44 mitogen-activated protein (MAP) kinase (MEK) → p42/p44 MAP kinase (ERK 1 and 2). Recent reports suggest that activation of this Ras/MAP kinase pathway is critical for the effects of insulin on mitogenesis and c-fos transcription but is not required for insulin action on metabolic processes such as glucose synthesis, lipogenesis, and Glut-4-mediated glucose transport. We have used three distinct approaches to examine the role of the Ras/MAP kinase pathway in the regulation of PEPCK transcription by insulin in H4IE-derived liver cells: (i) chemical inhibition of Ras farnesylation, (ii) infection of cells with an adenovirus vector encoding a dominant-negative mutant of Ras, and (iii) use of a chemical inhibitor of MEK. Although each of these methods blocks insulin activation of MAP kinase, none alters insulin antagonism of cAMP- and glucocorticoid-stimulated PEPCK transcription. Although phorbol esters activate MAP kinase and mimic the effects of insulin on PEPCK gene transcription, inhibition of MEK has no effect on phorbol ester inhibition of PEPCK gene transcription. Using the structurally and mechanistically distinct phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors, wortmannin and LY 294002, we provide further evidence supporting a role for PI 3-kinase activation in the regulation of PEPCK gene transcription by insulin. We conclude that neither insulin nor phorbol ester regulation of PEPCK gene transcription requires activation of the Ras/MAP kinase pathway and that insulin signaling to the PEPCK promoter is dependent on PI 3-kinase activation.

A major physiological function of insulin is the maintenance of glucose homeostasis, and the liver plays a pivotal role in this regulation. In the fasted state, blood glucose levels are maintained through hepatic glucose output, and in noninsulin-dependent diabetes mellitus, fasting hyperglycemia develops predominantly as a result of unrestrained hepatic gluconeogenesis (1–3). The enzyme phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) catalyzes the rate-limiting step in gluconeogenesis. Increased hepatic PEPCK expression has been demonstrated in several animal models of diabetes (4), and overexpression of PEPCK in transgenic mice results in the phenotype of noninsulin-dependent diabetes mellitus (5). There are no known allosteric modifiers of PEPCK; its activity is regulated by the level of gene expression (4, 6). Insulin and phorbol esters inhibit basal PEPCK gene transcription and antagonize the induction of PEPCK expression by glucocorticoids and glucagon (or its second messenger cAMP) (4, 7). Although insulin regulates the transcription of many genes, the regulation of PEPCK transcription has been the most extensively studied and has served as a useful model (6).

The signaling pathways involved in insulin action have been the subject of intense research (for review see Refs. 8 and 9). Ligand binding stimulates insulin receptor-mediated tyrosine phosphorylation of IRS-1 and Shc. These molecules then function as high affinity binding sites for several downstream effectors through src homology 2 domains. The two best studied effectors that bind to the IRS-1 docking protein are PI 3-kinase and Grb-2-Sos. The mechanism through which PI 3-kinase activation by insulin or other agents leads to biologic effects is poorly understood. Phosphorylated inositol products have been proposed to activate specific phorbol ester-insensitive protein kinase C isoforms (10, 11), and recently a serine/threonine protein kinase encoded by the Akt proto-oncogene was identified as a novel target of PI 3-kinase-generated lipids (12). PI 3-kinase also appears to be tightly associated with or contain a protein kinase activity (13, 14). Whatever the downstream mechanisms, studies based on PI 3-kinase inhibition have provided clear evidence for a role of this enzyme in the effect of insulin on Glut-4-mediated glucose transport (15, 16), antilipolysis (15, 17), c-fos expression (18, 19), mitogenesis (18, 20), glycogen synthase kinase-3 (21, 22), glycogen synthesis (23–25), amino acid transport (20), and membrane ruffling (26).

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† The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; PI, phosphatidylinositol; MAP kinase, p42/p44 mitogen-activated protein kinase or ERK 1 and 2; MEK, ERK kinase or MAP kinase kinase; IRS-1, insulin receptor substrate-1; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; DME, Dulbecco’s modified Eagle’s medium; c-jun, stress-activated or c-jun N-terminal kinase.
The other major insulin signaling pathway that is initiated by src homology 2-dependent binding to IRS-1 is the Ras/MAP kinase cascade. Binding of the Grb-2-Sos adapter complex to IRS-1 (or Shc) activates Ras through a SOS-mediated GDP-GTP exchange. Ras subsequently stimulates Raf through a poorly understood mechanism requiring the recruitment of other as yet unidentified factors (27, 28). A linear phosphorylation cascade of Raf → MEK → p42/p44 MAP kinase (ERK 1 and 2) subsequently occurs. MAP kinase activation represents a major branch point, and this enzyme may translocate to the nucleus to activate several specific transcription factors. Phorbol esters (via stimulation of protein kinase C) also activate the MAP kinase cascade, and although the precise mechanism is unclear, protein kinase C activation of Raf has been implicated (29, 30).

Several approaches have been taken to study the role of the Ras/MAP kinase pathway in the metabolic effects of insulin. Cells have been transfected or microinjected with Ras mutants (19, 31–34) or a dominant-negative Sos (35), thereby blocking Ras activation by insulin. Adipocytes have been permeabilized to allow the entry of GTP analogs that inactivate Ras (36). Antisense oligonucleotides have also been used to block Raf activity (37). Clonally selected cells in which active Raf mutants were introduced by retroviral infection have also been studied (38). In addition, the ability in certain cells of other growth factors to stimulate the Ras/MAP kinase cascade has been compared with the selective metabolic effects of insulin in these cells (39–41). Recently, a specific chemical inhibitor of MEK has been developed (42), and its effects were studied in muscle and adipose cell lines (43). Collectively, these studies support a role for activation of the Ras/MAP kinase pathway in nuclear effects of insulin on mitogenesis and c-fos expression (19, 33, 34, 37), but no obligatory role has been established in stimulation of Glut-4-mediated glucose transport (31, 38), glycogen synthesis (23, 25, 35, 41, 43), or lipogenesis (40, 43). Evidence also indicates that the regulation of c-fos gene transcription by insulin requires both Raf and PI3-kinase (39). It is unknown whether the involvement of MEK in insulin’s actions on PEPCK gene transcription. We conclude that insulin regulation of PEPCK gene transcription does not require Ras/MAP kinase pathway activation but is dependent on PI 3-kinase activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The MEK inhibitor PD 98059 and the farnesyltransferase inhibitor PD 152440 were generously provided by Dr. Alan Saltiel (Parke-Davis Warner Lambert Pharmaceutical Research División, Ann Arbor, MI). The farnesyltransferase inhibitor BST1 and the Ras-dependent protein kinase inhibitor were obtained from Bachem Bioscience Inc. (King of Prussia, PA). The PI 3-kinase inhibitor LY 294002 was kindly provided by Dr. Bentley Cheatham (Joslin Diabetes Center, Boston, MA). Polyclonal anti CT-IRS-1 antibody was a gift from Dr. Morris White (Joslin Diabetes Center, Boston, MA). [3H]-ATP (700 Ci/mmol) was purchased from ICN, and the CAT enzyme-linked immunosorbent assay kit was from Boehringer Mannheim. All other reagents were obtained from Sigma.

**Cell Culture Conditions**—The isolation of the H4IIE rat hepatoma-derived stable transfectant, HLLC, which contains the PEPCK promoter sequence from −2100 to +69 ligated to the CAT reporter gene, has been described previously (46). This cell line has been extensively characterized and contains all the promoter elements required for maximal gene regulation by cAMP, dexamethasone, and insulin. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum (Life Technologies, Inc.), 5.5 mm glucose, 50 IU/ml penicillin, and 50 mg/ml streptomycin in a humidified 5% CO2 atmosphere. Hormone and inhibitor additions were carried out with cells at 70% confluence in 10-cm tissue culture plates for times and at the concentrations detailed in the figure legends. For CAT protein determinations, cells were harvested by trypsinization and lysis in 0.45 ml of CAT lysis buffer according to the method used for CAT measurements are expressed as the percentage of CAT protein relative to the amount obtained in the presence of cAMP and dexamethasone. Adenovirus rasAsn17 Mutant—Construct and Recombinant Adenovirus—A recombinant adenovirus expressing a dominant-negative Ras was generated by cloning of the cDNA with serine mutated to asparagine at position 17 (gift of G. Cooper) into the polylinker of the vector pACCMVpLPa (gift of C. Newgard) by a series of microinjection and transfection in well characterized liver cell lines.

Although the regulation of PEPCK is a critical physiological site of insulin action, little is understood regarding the initial signal transduction mechanisms involved. We have used three different approaches to examine the involvement of the Ras/MAP kinase pathway in insulin signaling to PEPCK gene transcription in H4IIE-derived liver cells. Farnesyltransferase is an obligate step in Ras processing, and the structural and functional properties of the Ras protein in the same manner as those of Ras (48), with the results being confirmed by the cigarette smokers (28). When the expression of the vector pACCMVpLPa-Asn17 was cotransfected in confluent 293 cells using the CaPO4-DNA coprecipitation technique with modified bovine serum (Stratagene kit 200388). After 8–10 days, the death of the 293 cells was used to select for recombinant virus encoding Ras Asn17 protein. The viral DNA was then extracted from 293 cells, and the insert was confirmed by Southern blotting analysis. A single clone of recombinant adeno virus was isolated through serial dilution using a plaque assay. The experiment was repeated twice with similar results. The recombinant adeno virus was subsequently subcloned in Xba1-PstI sites of the pGem3zf vector polylinker. The Ras Asn17 DNA was then isolated using the BamHI-PstI sites and cloned into the PBSL1-SK+ vector. The corresponding BamHI–SalI fragment was then finally cloned into the corresponding sites of the pACCMVpLPa polylinker with the generation of pACCMVpLPa-rasAsn17. The pACCMVpLPa-rasAsn17 vector and the pBl M17 vector (gift of C. Newgard) were cotransfected in confluent 293 cells using the CaPO4-DNA coprecipitation technique with modified bovine serum (Stratagene kit 200388). Upon treatment with hormone/insulin for the times and the concentrations indicated in the table legend, total cellular RNA (approximately 100 µg of cells) was isolated (46). Oligonucleotide complementary sequences were detected by hybridization to the original [45] report by demonstrating inhibition of PI 3-kinase activity at the wortmannin concentrations used to antagonize insulin’s actions on PEPCK gene transcription. We conclude that insulin regulation of PEPCK gene transcription does not require Ras/MAP kinase pathway activation but is dependent on PI 3-kinase activation.
Ras/MAP Kinase Signaling Pathway and PEPCK Gene Expression

+42 to +67, relative to the transcription start site, in the PEPCK and β-actin genes were then used in primer extension assays as described (45). Autoradiograms were subsequently quantified by PhosphorImager analysis (Molecular Dynamics).

Fig. 1. Inhibition of Ras or MEK blocks insulin stimulation of MAP kinase activity. H4IIE cells stably transfected with a PEPCK promoter CAT construct (HLIC, see "Experimental Procedures") were preincubated with the farnesyltransferase inhibitors (50 μM B581 and 10 μM PD 152440–0011B) or adenovirus containing the Asn17 dominant-negative mutant of Ras for 16 h in DMEM containing serum. Cells were subsequently washed twice with PBS and placed in serum-free DMEM prior to the addition of insulin. The MEK inhibitor PD 98059 (10 μM) was added 30 min prior to the addition of insulin to cells deprived of serum for 18 h. Cells preincubated with inhibitors as above were then treated with insulin (10 ng) for 5 min and subsequently analyzed for MAP kinase activity as described under "Experimental Procedures." The results are the means ± S.E. of three experiments.

Results
Elements of the insulin signaling pathway involved in the regulation of PEPCK gene transcription were studied in the rat hepatoma-derived cell line (H4IIE) stably transfected with the PEPCK promoter sequence from −2100 to +69 (relative to the transcriptional start site) ligated to a CAT reporter gene (termed HL1C cells) (46). This stable transfectant has been previously characterized by insulin, glucocorticoid, cAMP, and phorbol esters when compared with the endogenous gene. That is, insulin and phorbol esters act in a dominant fashion, blocking the induction of PEPCK-CAT transcription by cAMP and dexamethasone (46). Given the low basal level of PEPCK transcription in the absence of other effectors, transcriptional regulation of PEPCK has been best studied using the ability of insulin to counteract stimulation of gene transcription by glucagon (or cAMP) and glucocorticoids (4, 7, 45, 46, 51). A similar need to observe insulin effects as the antagonism of the actions of counter-regulatory hormones has been reported for many of insulin’s actions in liver. Here we focus on the signaling pathways that mediate insulin’s antagonism of maximally stimulatory concentrations of cAMP (using the nonhydrolyzable analog 8-CPT-cAMP) and the synthetic glucocorticoid dexamethasone.

Role of Ras in Insulin Regulation of PEPCK Gene Transcription—The role of Ras in insulin regulation of the PEPCK-CAT fusion gene was first investigated by blocking farnesylation of Ras (an obligatory step in Ras processing) using two structurally different farnesyltransferase inhibitors, B581 and PD 152440 (52, 53). The ability of these inhibitors to block Ras signaling in HLIC cells was assessed by studying their effects on insulin activation of MAP kinase. Pretreatment of the cells with either of the farnesyltransferase inhibitors completely blocked insulin stimulation of MAP kinase activity (Fig. 1), consistent with previous studies using these inhibitors in other cell systems (52).2 There was no effect of cAMP and dexamethasone on MAP kinase activity (data not shown).

The farnesyltransferase inhibitors B581 and PD 152440 had no effect on the ability of insulin to antagonize cAMP/dexamethasone induction of the PEPCK-CAT fusion gene (Fig. 2, A and B). Despite completely blocking insulin stimulation of MAP kinase, there was no change in the ability of either submaximal or maximal insulin concentrations to inhibit PEPCK-CAT expression (Fig. 2 and data not shown).

HLIC cells were infected with an adenovirus expressing a dominant negative mutant of ras (Asn17) to assess the role of this component of the signaling pathway. Whereas infection with this adenovirus completely blocked insulin stimulation of MAP kinase activity (Fig. 1), it had no effect on insulin inhibition of PEPCK-CAT gene expression (Fig. 3). The same adenovirus containing a β-galactosidase gene instead of the mutant

2 A. Saltiel and J. Sebolt-Leopold, personal communication.
Ras had no effect on insulin stimulation of MAP kinase or insulin action on PEPCK-CAT gene expression (data not shown). These results indicate that insulin signaling to the PEPCK promoter does not require the activation of Ras.

Interestingly, either mechanism of Ras inhibition (farnesyl-transferase inhibition or expression of dominant-negative Ras) augments the effects of cAMP and dexamethasone on PEPCK-CAT gene expression. This response is the result of a superinduction of the dexamethasone response (data not shown), possibly due to an effect of basal Ras activity to tonically inhibit dexamethasone action. Whatever the mechanism, however, insulin was clearly capable of antagonizing the synergistic induction of PEPCK-CAT transcription by cAMP and dexamethasone in the presence or the absence of Ras inhibition (Figs. 2 and 3). Insulin also blocked the induction of PEPCK-CAT expression by dexamethasone alone in the presence of the farnesyltransferase inhibitor B581 (data not shown).

Role of MEK Activation in Insulin and Phorbol Ester Regulation of PEPCK Gene Transcription—Ras activation leads to Raf and subsequently MEK activation; however, some Ras-independent mechanisms for the activation of MAP kinase have been described (54–56). Recently a specific inhibitor of MEK (PD 98059) was identified that blocks insulin induction of c-fos transcription in both 3T3 adipocytes and L6 myocytes (42, 43). PD 98059 completely blocked insulin activation of MAP kinase in HL1C cells (Fig. 1) but again did not interfere with insulin action on the PEPCK-CAT fusion gene (Fig. 4). Similar results were obtained looking at the effects of MEK inhibition on insulin regulation of endogenous PEPCK gene expression (Fig. 5). These results indicate that MEK is not required for insulin signaling to the PEPCK gene promoter. Interestingly, unlike Ras blockade, MEK inhibition does not augment the action of cAMP and dexamethasone on PEPCK-CAT expression. It appears that augmentation of the dexamethasone response by Ras/MAP kinase pathway inhibition requires changes that occur over a longer time course. The MEK inhibitor was added for 30 min prior to hormone addition, as compared with 16 h incubations for the farnesyltransferase inhibitors or Asn17 ras containing adenovirus. Prolonged treatment of cells with the MEK inhibitor (16 h) does in fact cause an equivalent augmentation of the dexamethasone response (data not shown).

Phorbol esters mimic the effects of insulin on PEPCK gene transcription in this system by acting in part through the same promoter sequence (44). Activation of protein kinase C by phor-
bolesters can stimulate the MAP kinase signaling cascade, and it has been suggested that this may occur through protein kinase C activation of Raf, which lies immediately upstream of MEK (29, 30). Consequently, the effects of MEK inhibition on phorbol ester (PMA) signaling to PEPCK gene transcription were examined. Interestingly, MEK inhibition had no effect on PMA regulation of either the PEPCK-CAT fusion gene (Fig. 4) or the endogenous PEPCK gene (Fig. 5). Similarly, inhibition of Ras using either of the farnesyltransferase inhibitors did not alter phorbol ester mediated inhibition of PEPCK-CAT gene expression (data not shown). These results indicate that phorbol esters, like insulin, inhibit PEPCK gene transcription through a mechanism independent of Ras/MAP kinase pathway activation.

Role of PI 3-Kinase Activation—An important role for PI 3-kinase activation in the regulation of PEPCK transcription by insulin was recently demonstrated using the selective PI-3-kinase inhibitor wortmannin (45). Wortmannin inactivates PI 3-kinase by covalently binding to the p110 kDa subunit, whereas at higher concentrations it can also inhibit myosin light chain kinase (57). Most studies with wortmannin have involved short incubation times (<30 min) because this compound has been shown to lose effectiveness after approximately 2 h of incubation at physiological pH (58, 59). Relatively high concentrations of wortmannin (500 nM) were used for the much longer incubation times (>3 h) required to measure changes of PEPCK gene transcription in the initial studies (45). Here a concentration of wortmannin where specificity is maintained (100 nM) was used and re-added after 2 h (based on the described half-life for this compound in pH 7.4 buffered medium (58, 59)). The effects of wortmannin were also compared with those of the more stable and mechanistically distinct PI 3-kinase inhibitor LY 290042. The structurally unrelated LY 294002 competes with ATP for binding to the p110 subunit (60). LY 294002 and to a lesser extent wortmannin augmented the induction of PEPCK-CAT gene expression by cAMP/dexamethasone (Fig. 6) due to enhancement of the dexamethasone response (data not shown). This suggests that perhaps inhibition of basal PI 3-kinase activity may remove some tonic inhibition of the dexamethasone response. However, the augmentation of the cAMP/dexamethasone effect seen with wortmannin was not observed when the endogenous gene was studied (45).

Both wortmannin and LY 290042 blunted the effect of insulin on inhibition of PEPCK-CAT gene expression (Fig. 6). Insulin-stimulated PI 3-kinase activity was blocked in the presence of 100 nM wortmannin (Fig. 7). Dexamethasone and cAMP had no effect on PI 3-kinase activity (data not shown). Therefore, using two structurally and mechanistically distinct inhibitors of PI 3-kinase, we demonstrate an important role for PI 3-kinase in insulin regulation of PEPCK gene transcription.

DISCUSSION
Liver, a key target of insulin action, controls fasting blood glucose primarily by regulating the rate of gluconeogenesis. The rate of gluconeogenesis is controlled in large part by changes in the transcription of the rate-limiting enzyme in this pathway, PEPCK (4, 6). Although there has been a great deal
activation plays an important role in insulin regulation of PEPCK gene transcription. Recently, wortmannin has been used to show that PI 3-kinase is involved in the early components of the insulin signal transduction pathway responsible for the effects of insulin on PEPCK gene transcription (45). Activation of the Ras → Raf → MEK → MAP kinase pathway is another major component of insulin signal transduction. Previous studies have suggested that Ras pathway activation plays a pivotal role in the effects of insulin on mitogenesis and c-fos expression (19, 33, 34, 37).

Fig. 6. PI 3-kinase inhibition diminishes insulin signaling to the stably transfected PEPCK-CAT fusion gene. The PI 3-kinase inhibitor LY 294002 (50 μM) was added to HL1C cells (deprived of serum for 18 h) 15 min prior to the addition of hormones. Wortmannin (100 nM) was also added to serum-deprived cells 15 min prior to hormone additions, and at 2 h 100 nM wortmannin was re-added. In all cases, 4 h after hormone addition, cells were analyzed for CAT protein as described under “Experimental Procedures.” Hormone additions were as follows: no addition (basal), 0.1 mM 8-CPT-cAMP and 500 nM dexamethasone (cAMP/Dex) or 8-CPT-cAMP, dexamethasone, and insulin at the indicated concentrations (0.5, 1, of 10 nM). The results are expressed as the percentage of CAT protein relative to cAMP/Dex-stimulated CAT protein in the absence of wortmannin or LY 294002 and represent the means ± S.E. of four separate experiments.

Fig. 7. Wortmannin blocks insulin stimulation of PI 3-kinase activity in HL1C cells. Serum-deprived cells were incubated with 100 nM wortmannin or Me2SO carrier for 15 min and subsequently 10 nM insulin was added for 5 min. PI 3-kinase activity was measured in cell extracts as described under “Experimental Procedures,” and the products were analyzed by thin layer chromatography. An autoradiograph of a representative experiment is shown. The location of the reaction product, phosphatidylinositol-3-phosphate (P13P), is indicated.

of progress in identifying the cis-acting elements that mediate the effects of insulin on PEPCK gene transcription, little is known regarding the early components of the insulin signal transduction pathway responsible for this action of insulin. Recently, wortmannin has been used to show that PI 3-kinase activation plays an important role in insulin regulation of PEPCK gene transcription (45). Activation of the Ras → Raf → MEK → MAP kinase pathway is another major component of insulin signal transduction. Previous studies have suggested that Ras pathway activation plays a pivotal role in the effects of insulin on mitogenesis and c-fos expression (19, 33, 34, 37) but may not be required for many of the classic metabolic effects of insulin such as stimulation of glycogen synthesis (23, 25, 35, 40, 41), Glut-4-mediated glucose transport (31, 38), and lipogenesis (40, 43). Despite the importance of the liver in glucose homeostasis and the extensive scrutiny of the Ras/MAP kinase cascade in many systems, the only attempt to study the role of this pathway in hepatic insulin action has focused on the regulation of mitogenesis (37). For this reason we examined the involvement of the Ras/MAP kinase pathway in the regulation of PEPCK gene transcription by insulin. We describe the use of three mechanistically distinct approaches to inhibit the Ras/MAP kinase pathway in liver (farnesyltransferase inhibition, dominant-negative Ras adenovirus infection, and a chemical inhibitor of MEK) and demonstrate that they all fail to alter the ability of insulin to regulate PEPCK gene transcription.

Ras requires post-translational farnesylation to localize it to the plasma membrane. Recently, farnesyltransferase inhibitors have been developed with the goal of blocking Ras transformation (61). A new specific peptidomimetic farnesyltransferase inhibitor, B581, which mimics the CAAX binding site of the farnesyltransferase enzyme, blocks Ras transformation and activation of MAP kinase, a downstream target of Ras activation (52). We have used this inhibitor and a structurally distinct analog (PD 152440) to assess the role of the Ras/MAP kinase cascade in the regulation of PEPCK gene transcription by insulin. Lovastatin has been the only other farnesyltransferase inhibitor used to study the role of Ras in insulin signaling (62). Unfortunately, lovastatin has several sites of action apart from inhibiting farnesylation including inhibition of the rate-limiting step of cholesterol biosynthesis. Lovastatin can also block cell transformation by Raf (63), a downstream target of Ras, and decrease insulin activation of PI 3-kinase activity in Rat-1 fibroblasts (64). Lovastatin inhibition of PI 3-kinase activity may account for its ability to block the effects of insulin on glycogen synthesis (62), a process that appears to be dependent on PI 3-kinase activation (23–25) but not Ras/MAP kinase pathway activation (25, 35, 43). The farnesyltransferase inhibitors used in this study, B581 and PD 152440, are designed to specifically target farnesyltransferase activity without affecting cholesterol biosynthesis (52, 53). B581 prevents Ras but not Raf transformation (52). It is unlikely that they significantly inhibit insulin activation of PI 3-kinase because PI 3-kinase inhibition does diminish the insulin response on PEPCK transcription (Figs. 1 and 45). Although these inhibitors block insulin activation of MAP kinase, they fail to alter insulin’s ability to suppress PEPCK gene transcription (Figs. 1 and 2).

The introduction of genes into mammalian cells has sometimes been difficult because many cell types are difficult to transfect with the high efficiency necessary to observe the effects of overexpression or inhibition. This problem has been partially circumvented by the use of retroviral vectors; however, these require integration of viral DNA into genomic DNA during cell division, often over several days during which a variety of adaptive changes may occur. Recently, adenoviral vectors have been used to introduce genes of interest into mammalian cells and tissues for metabolic studies (48). This approach allows high efficiency expression of genes within several hours, minimizing cellular adaptation to these genetic changes. Here we have used an adenovirus vector encoding a dominant negative mutant of ras (Asn17) to demonstrate that Ras is not required for the regulation of PEPCK gene expression by insulin.

In addition to direct inhibition of Ras, we studied the effects of blocking activation of a downstream component of the Ras/MAP kinase cascade. Although the Ras cascade of Ras → Raf → MEK → MAP kinase is believed to be a linear series of activa-
tions, some Ras-independent mechanisms of MEK and MAP kinase activation have been proposed (54–56). Recently, a specific chemical inhibitor of MEK has been developed and used in 3T3 adipocytes, L6 myocytes, and PC12 cells to specifically inhibit MAP kinase activation without any measurable effect on a variety of other kinases, including the insulin receptor kinase, protein kinase C, cAMP-dependent protein kinase, and PI 3-kinase (42, 43). Consistent with results obtained following Ras inhibition, the MEK inhibitor, PD 98059, did not inhibit the ability of insulin to decrease PEPCK gene transcription (Figs. 4 and 5).

Phorbol esters activate protein kinase C and mimic the effects of insulin on PEPCK gene transcription. Phorbol esters also stimulate the MAP kinase cascade in many cell types. This appears to occur through activation of Raf because dominant negative mutants of Raf block the stimulation of MAP kinase by PMA (29). Here we demonstrate that inhibition of the Ras pathway either upstream of Raf (through inhibition of Ras farnesylation) or downstream of Raf (through MEK inhibition) fails to alter PMA inhibition of PEPCK gene transcription. Interestingly, the PI 3-kinase inhibitor wortmannin attenuated insulin but not PMA action on PEPCK gene transcription (45), although the signals generated by these agents converge at the same cis-acting DNA element within the PEPCK promoter (44). These data suggest that signaling from PMA-stimulated protein kinase C isosforms to the PEPCK gene promoter is through some molecule independent of both the Ras/MAP kinase pathway and PI 3-kinase activation.

Although mounting evidence suggests that the Ras/MAP kinase pathway is not required for many of the classic metabolic actions of insulin, a clear role for PI 3-kinase activation in these pathways has emerged. PI 3-kinase activation by insulin appears to be required for insulin effects on Glut-4-mediated glucose transport (15, 16), glycogen synthesis (23–25), anti-lipolysis (15, 17), amino acid transport (20), membrane ruffling (26), mitogenesis (16, 18, 20), and c-fos expression (18, 19). Recently, an important role for PI 3-kinase activation in insulin regulation of PEPCK transcription was demonstrated using the selective PI 3-kinase inhibitor wortmannin (45). Here we extend these results by showing that lower concentrations of wortmannin (100 nM), followed by a re-addition at 2 h (to compensate for the compound’s instability (58, 59)), also diminishes the effect of insulin on PEPCK transcription. This concentration of wortmannin completely blocked stimulation of PI 3-kinase activity by insulin (Fig. 7). The results obtained with LY 290042, which has a unique mechanism of action, supports those noted with wortmannin (Fig. 6). This adds the regulation of PEPCK gene transcription to the growing list of wortmannin/LY 290042-sensitive pathways. Based on the requirement of PI 3-kinase but not Ras/MAP kinase pathway activation, it appears that the mechanism of insulin regulation of PEPCK gene transcription is more analogous to the regulation of other classic metabolic actions of insulin (i.e. glucose transport, glycogen synthesis, lipogenesis) and differs from that involved in the mitogenic/growth-promoting actions of insulin, which require both PI 3-kinase and Ras/MAP kinase pathway activation.

Inhibition of either the Ras/MAP kinase pathway or PI 3-kinase augments the ability of dexamethasone to stimulate PEPCK-CAT fusion gene expression (Figs. 2, 3, and 4 and data not shown). Whether these effects are due to inhibition of constitutively active Ras, MEK, or PI 3-kinase activities, a direct effect on dexamethasone signaling, or some other mechanism is unclear. In Chinese hamster ovary cells overexpressing the insulin receptors (35) and H4IIE hepatoma cells (37), Ras pathway inhibition diminished basal mitogenesis. Thus there is evidence for constitutive activity of the Ras/MAP kinase pathway. In addition, basal transcription of the collagenase gene is also reduced by Ras inhibition (33). However, the ability of wortmannin to augment the dexamethasone response was not observed when endogenous PEPCK mRNA was measured (45). The mechanism underlying this augmentation of dexamethasone action on PEPCK-CAT fusion gene expression remains to be determined.

Insulin regulates the transcription of many genes (for review, see Ref. 6); however, participation of the Ras pathway in these effects of insulin has only been studied with regards to c-fos and collagenase gene regulation (19, 33, 34). The MEK inhibitor, PD 98059, was used to delineate the obligatory role of Ras in the regulation of c-fos transcription (43) confirming earlier reports using ras mutants in cell transfection and microinjection experiments (19, 33, 34). Likewise insulin activation of a collagenase promoter-CAT construct was suppressed by transfection with a dominant negative ras mutant (33). In contrast we now demonstrate that the regulation of PEPCK gene transcription by insulin is not dependent on Ras/MAP kinase pathway activation. Whether this observation represents a unique transcriptional signaling mechanism in liver, a mechanism unique to the PEPCK gene, or a common mechanism for insulin regulation of gene expression remains to be resolved. There is emerging evidence that many signaling events and mechanisms of insulin action may be tissue-specific. For example, Ras does not appear to be required for insulin stimulation of Glut-4-mediated glucose transport in adipose cell lines (31, 38) but may be important in cardiac myocytes (32). There are also suggestions that glycogen synthase is regulated differently in muscle (65) and other cell types (i.e. Chinese hamster ovary cells) (35). In addition, Shc, an alternative substrate for the insulin receptor tyrosine kinase, appears to play a predominant role in Rat1 fibroblasts (66), whereas this does not appear to be true in PC12 cells (67) or in intact liver (68). These tissue-dependent differences make further investigation of insulin signaling in metabolically important tissues, such as liver, of great interest.

A potential Ras independent pathway that may explain the ability of insulin and PMA to repress PEPCK transcription is the stress-activated or c-jun N-terminal kinase pathway (J NK). J NK, a member of the MAP kinase superfamily, can be activated independent of the Ras → Raf → MEK → MAP kinase cascade (69, 70), possibly involving other members of the Ras GTPase superfamily (70, 71). J NK appears to act through the regulation of specific transcription events (72). In Chinese hamster ovary cells overexpressing insulin receptors, however, insulin does not appear to stimulate J NK activity. Further studies will be necessary to clarify the role of J NK in the regulation of transcription by insulin.

In conclusion, we have used several approaches to study the role of the Ras pathway in liver cells: inhibition of Ras farnesylation, adenovirus infection with a Ras dominant-negative mutant, and inhibition of MEK. All three methods block insulin stimulation of MAP kinase, but none affect insulin action on PEPCK gene transcription. We conclude that insulin regulation of PEPCK gene expression does not require Ras/MAP kinase pathway activation but is dependent on PI 3-kinase stimulation. Similar approaches will be readily applied to delineating the role of the Ras cascade in other aspects of liver metabolism.

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Insulin Regulation of Phosphoenolpyruvate Carboxykinase Gene Expression Does Not Require Activation of the Ras/Mitogen-activated Protein Kinase Signaling Pathway

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