Activation of the Ras Mitogen-activated Protein Kinase-Ribosomal Protein Kinase Pathway Is Not Required for the Repression of Phosphoenolpyruvate Carboxykinase Gene Transcription by Insulin

Calum Sutherland, Mary Waltner-Law, Luigi Gnucci, Barbara B. Kahn, and Daryl K. Granner

From the Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37222-0615 and the Diabetes Unit, Division of Endocrinology and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first committed step in hepatic gluconeogenesis. Glucagon and glucocorticoids stimulate PEPCK gene transcription, whereas insulin has a dominant inhibitory effect. We have shown that inhibitors of 1-phosphatidylinositol 3-kinase (PI 3-kinase) block this action of insulin. In contrast, three distinct agents, all of which prevent activation of p42/p44 mitogen-activated protein (MAP) kinase, have no effect on the regulation of PEPCK transcription by insulin. However, a subsequent report has suggested that this pathway is involved in the inhibition of cAMP-induced PEPCK gene transcription by insulin. To address these conflicting data, we re-examined the Ras MAP kinase pathway, not only with respect to regulation of PEPCK gene transcription, but also for regulation of PI 3-kinase and p42/p44 MAP kinase. Overexpression of constitutively active Ras (V61) (or Raf-1 (RafCAAX)) partially represses PEPCK transcription in hepatoma cells. However, an inhibitor of MAP kinase blocks this action of RafCAAX but has no effect on regulation of PEPCK gene transcription by insulin. Second, the action of a dominant negative Ras (N17Ras) on PEPCK gene transcription correlates more closely with the inhibition of PI 3-kinase than with the inhibition of p42/p44 MAP kinase. Third, insulin cannot activate p42/p44 MAP kinase in the presence of cAMP even though cAMP-induced PEPCK gene transcription is inhibited by insulin. This data confirms that the Ras MAP kinase pathway is not required for the regulation of PEPCK gene transcription by insulin and demonstrates the importance of employing multiple techniques when investigating the function of signaling pathways.

Insulin regulates the expression of more than 100 genes. Regulation of expression can be at the level of gene transcription, mRNA stability, translation, or protein degradation. The key hepatic gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK)1 is one of the best studied insulin-regulated genes, and its expression is regulated primarily at the level of gene transcription (1, 2). In rat liver or in the H4IIE rat hepatoma cell line, cAMP, glucocorticoids, and retinoic acid all stimulate transcription of the PEPCK gene (for review see Refs. 1 and 2). In contrast, insulin, glucose, phorbol esters, hydrogen peroxide, or sodium arsenite repress cAMP, glucocorticoid, or retinoic acid stimulation of this gene in a dominant fashion (3–6). Whereas the complex glucocorticoid response unit that mediates the stimulatory action of glucocorticoids (7, 8) and the multiple cis-acting elements required for cAMP-stimulated PEPCK gene transcription (9) have been well characterized, the molecular mechanisms that mediate the repression of transcription by insulin are less well defined. An insulin response sequence (IRS) positioned between –413 and –407 mediates the repression of PEPCK gene transcription by insulin in stably transfected cell lines (10) and in a heterologous context (11). This sequence mediates both an insulin and a phorbol ester response within the PEPCK promoter (12). A second IRS (that equally contributes to the total insulin response) is located more proximal to the transcription start site of the promoter, although its precise location has not yet been determined (10).

Similarly, although the signaling mechanisms employed to induce PEPCK gene transcription by glucocorticoids, retinoid acid, and glucagon are relatively well characterized, the signaling pathways leading to repression of this gene are only now beginning to be elucidated (6, 13, 14). Interestingly, it is becoming clear that insulin uses distinct signaling pathways to regulate the activity of different gene promoters (1, 15, 16). For example, the regulation of expression of certain immediate early genes requires activation of the Ras MAP kinase pathway (1, 17), whereas the regulation of PEPCK gene expression in hepatoma cells by insulin is sensitive to inhibitors of PI 3-kinase but not to either rapamycin (an inhibitor of p70S6 kinase activation) or inhibitors of the Ras MAP kinase pathway (13, 14). However, the induction of hexokinase II gene expression in myotubes by insulin is blocked by both PI 3-kinase inhibitors and rapamycin but not inhibitors of the Ras MAP kinase pathway (16). Recently, in contrast to the previous work, another

1 The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase (GTP-oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32); IRS-1, insulin receptor substrate 1; PI 3-kinase, phosphatidylinositol 3-kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase and extracellular-regulated kinase kinase; p60RSK, p90 ribosomal protein kinase; GR, glucocorticoid receptor; PKA, cAMP-dependent protein kinase; S6K, p70S6 kinase; eIF4E, eukaryotic release factor-3; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; 8CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; CAT, chloramphenicol acetyltransferase; CBP, CREB response element binding protein; DMEM, Dulbecco’s modified Eagle’s medium; adN17Ras, adenovirus N17Ras.
Insulin Repression of PEPCK Transcription

The group described a loss of insulin repression of PEPCK gene transcription in the presence of a dominant negative Ras mutant (N17Ras) (18). The existence of an insulin signaling pathway to the PEPCK gene that requires activation of the Ras MAP kinase pathway and the subsequent translocation of the p90 ribosomal S6 protein kinase (p90Rsk) to the nucleus was proposed. p90Rsk is postulated to interact with the co-activator molecule CREB binding protein (CBP/p300), thereby blocking the ability of cAMP to regulate gene transcription through CBP/p300. The CBP/p300 protein has been implicated in the binding of many proteins and could have a role in the regulation of transcription of multiple genes by distinct effectors (for review see Refs. 19–21).

As this scenario is contrary to our previous work, which dissociated the Ras MAP kinase pathway from regulation of PEPCK gene transcription by insulin (13, 14), we decided to re-examine the role of this pathway in the regulation of PEPCK gene transcription to attempt to determine the basis for this discrepancy. We determined that the inhibitor of the Ras MAP kinase pathway employed by Nakajima et al. (18) can affect the activity of the PI 3-kinase pathway as well as the MAP kinase pathway if it is expressed at high enough levels. This action of the inhibitor could explain the apparently discordant results. In addition, we present further evidence that eliminates the Ras MAP kinase pathway as the mediator of insulin repression of PEPCK gene transcription, even though the activation of this pathway can partially repress PEPCK gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Lysis, and CAT Assay—The H4IIE rat hepatoma-derived stable transfectant, HL1C, contains the PEPCK promoter sequence from −2100 to +69 ligated to the CAT reporter gene (10). HL1C cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5.5% fetal calf serum and 2.5% newborn calf serum. Hormone and inhibitor treatments were carried out in serum-free medium for the times and at the concentrations indicated in the figure legends. Cells were harvested by trypsin digestion and then sonicated in 400 μl of 250 mM Tris/HCl (pH 7.8) before CAT activity was determined (10).

RNA Isolation and Primer Extension Analysis—HL1C cells were serum-starved overnight and treated with hormone/inhibitor for the times and at the concentrations indicated in the figure legends. Total cellular RNA (~100 μg/106 cells) was isolated using Tri-Reagent (Molecular Research Center Inc.) according to the supplier's instructions. A primer extension assay was used to analyze PEPCK and β-actin mRNA accumulation as described previously (10), and autoradiographs were quantified by PhosphorImager analysis (Molecular Dynamics).

Preparation of Cell Extract for Kinase Assays—HL1C cells (2–4 × 106) were incubated in serum-free medium with hormones and inhibitors at the concentrations indicated in the figure legends. Cells were then scraped into 0.5 ml of ice-cold lysis buffer (25 mM Tris/HCl [pH 7.4], 50 mM NaF, 100 mM NaCl, 1 mM sodium vanadate, 5 mM EGTA, 1 mM EDTA, 1% (v/v) Nonidet P-40, 10 mM sodium pyrophosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 5% (v/v) glycerol, and 0.1% (v/v) 2-mercaptoethanol). Cell debris was removed by centrifugation at 13,000 × g for 5 min, and the protein concentration was determined by the method of Bradford using bovine serum albumin as an internal standard.

MAP Kinase Assay—After incubation with or without insulin for 15 min, cells were lysed in 500 μl of lysis buffer (as above) and cleared by centrifugation, and p42/p44 MAP kinase was immunoprecipitated using anti-extracellular-regulated kinase 1/2 antibodies (Santa Cruz). The precipitated kinase was assayed using myelin basic protein as a substrate (16).

PI 3-Kinase Assay—After incubation with or without insulin for 15 min, cells were lysed in 500 μl of lysis buffer (as above) and cleared by centrifugation. ActiNated PI 3-kinase was immunoprecipitated using an anti-IRS-1 antibody (Santa Cruz). The precipitated kinase was assayed using phosphatidylinositol as substrate in the presence of phosphatidylserine, MgCl2 (10 μM) and γ32P-ATP (50 μM, 10 μCi) for 15 min at 37 °C. The products were separated by TLC using an ammonium running system (methanol:chloroform:ammonia:water 20:14:3:5), and the radiolabeled PI 3-phosphate was visualized by autoradiography and/or quantified by PhosphorImager (Molecular Dynamics).

DNA Constructs and Adenovirus—The preparation of the PEPCK promoter-CAT reporter construct (pPL32) has been described previously (22), the constitutively active Ras (V61Ras) construct was a generous gift from Dr. Montminy (Boston, MA). A constitutively active Raf (RafCAAX) construct was a generous gift from Dr. David Stokoe (San Francisco, CA (23)). The preparation of the adenovirus-expressing dominant negative N17Ras has been described previously (14). Plaque-forming unit calculations were determined by measuring A405 (0.2 μg/ml) = 1 represents 1012 plaque-forming units/ml.

Transient Transfection—Various combinations (as indicated in the figure legends) of plasmids encoding PEPCK promoter-CAT reporter (pPL32 (22)), active Ras (V61Ras), active Raf-1 (RafCAAX), PKA catalytic subunit (PKA from R. Maurer, University of Oregon), glucocorticoid receptor (RSV-GR, from Keith Yamamoto, San Francisco), and control (RSV-neo) were transiently transfected into H4IIE cells as described previously (24). Cells were incubated for 20 h with hormones as indicated in the figure legends and then harvested by trypptic digestion and sonicated in 200 μl of 250 mM Tris/HCl (pH 7.5) before CAT activity was determined (10).

RESULTS

It has been proposed that the Ras MAP kinase pathway is required for insulin repression of cAMP-induced PEPCK gene transcription (18). This is in contrast to a previous study where expression of a dominant negative mutant of Ras (N17Ras), using an adenovirus expression system (adN17Ras), had no effect on the regulation of PEPCK gene transcription by insulin (14). This particular Ras mutant had no effect on PEPCK gene transcription when expressed at a level sufficient to completely inhibit the activation of p42/p44 MAP kinase by insulin (14). To resolve this disparity, we re-examined the effect of this mutant Ras on the regulation of PEPCK-CAT fusion gene expression in a stably transfected hepatoma cell line (HL1C) as well as on the regulation of endogenous PEPCK gene transcription (Fig. 1). There is no effect of adN17Ras on the hormone responsiveness of the PEPCK promoter when cells are treated with an amount sufficient to completely inhibit p42/p44 MAP kinase activation (Fig. 1, N17Ras-5 μl and Fig. 2). However, at much higher levels of adN17Ras treatment, the ability of insulin to repress PEPCK gene transcription is indeed partially lost (Fig. 1, N17Ras-20 μl). The extremely high efficiency of DNA transfer is a major advantage of using an adenovirus transfection system. The efficiency of DNA transfer in H4IIE cells increases from <0.1% with conventional transfection techniques to 70–100% with adenovirus treatment.2 The efficient transfer provided by the latter allows for the measurement of multiple endogenous parameters in each experiment. Thus we were able to determine the activities of p42/p44 MAP kinase and PI 3-kinase (Fig. 2) in parallel with the measurement of PEPCK gene expression (Figs. 1 and 2). The level of adN17Ras treatment required for inhibition of insulin action on PEPCK gene transcription is much higher than that required for complete inhibition of p42/p44 MAP kinase induction (Fig. 2) but does produce partial inhibition of PI 3-kinase activity (Fig. 2). In fact, the effect of N17Ras expression on PEPCK gene transcription correlates more closely with its effect on PI 3-kinase activity rather than with its effect on p42/p44 MAP kinase activity (Fig. 2). Thus the effect of N17Ras on PEPCK transcription reported elsewhere (18) may be due to inhibition of PI 3-kinase rather than the MAP kinase pathway (Fig. 2).

Interestingly, in most cell types studied, activation of p42/p44 MAP kinase is antagonized by agents that increase intracellular cAMP (25–29). One such study demonstrated that elevated cAMP blocks hepatocyte growth factor-induced MAP kinase activity in isolated rat hepatocytes (30). Thus, it was important to determine whether this kinase could be activated.

2. C. Sutherland and D. K. Granner, unpublished observations.
by insulin under the hormonal conditions used to examine the regulation of PEPCK gene transcription. Indeed, in H4IIE cells, p42/p44 MAP kinase is not activated by insulin in the presence of 8CPT-cAMP (Fig. 3). The repression of PEPCK gene transcription by insulin is dominant over the induction of transcription by cAMP (1). This is strong evidence that activation of the p42/p44 MAP kinase pathway cannot mediate the repression of cAMP-induced PEPCK gene transcription by insulin.

The results presented above (and previously (13, 14)) suggest that PI 3-kinase but not p42/p44 MAP kinase is required for the repression of the PEPCK promoter by insulin. However this does not preclude the possibility that activation of the Ras MAP kinase pathway is sufficient for repression of PEPCK gene transcription. To address this possibility, a plasmid encoding oncogenic Ras (V61Ras, kindly donated by Dr. M. Montminy) and a PEPCK promoter-CAT reporter fusion gene construct (pPL32, containing −468 to +69, relative to the transcription start site) were coexpressed in H4IIE cells. V61Ras expression partially reduces the induction of this PEPCK-CAT fusion gene by glucocorticoids or cAMP (PKA catalytic subunit, Fig. 4A). In addition, the expression of an active mutant of c-Raf (Raf-
CAAX), a protein kinase that lies downstream of Ras on the p42/p44 MAP kinase activation pathway, also partly represses PEPCK gene transcription (Fig. 4B). Thus, strong activation of the Ras-Raf MAP kinase pathway does appear to repress PEPCK gene transcription. However, an inhibitor of MEK, the kinase that links Raf to p42/p44 MAP kinase (Fig. 5), blocks the action of RafCAAX on PEPCK gene transcription without affecting the action of insulin on this gene (Fig. 4C). Again, this is consistent with the idea that the Ras-Raf-MAP kinase pathway is not required for the regulation of PEPCK gene transcription by insulin.

**DISCUSSION**

The delineation of signal transduction pathways routinely involves the use of dominant negative molecules, selective inhibitors of enzymes and expression of active mutants. Each method has inherent drawbacks. The specificity of inhibitors can be questioned. Dominant negative forms of signaling molecules are usually inactive mutants of a protein that are presumed to sequester an interacting molecule (upstream or downstream in the pathway), thus blocking the normal function of the endogenous signaling protein. This mode of action cannot preclude the possibility that an effect of a dominant negative protein predicts a role for the binding protein rather than the molecule itself. The overexpression of a constitutively active form of a signaling molecule can produce a loss of substrate specificity. In addition, many signaling pathways are activated transiently by a given agent and in a more sustained fashion by a second agent (e.g. epidermal growth factor and...
nerve growth factor activation of p42/p44 MAP kinase in PC12 cells (31)). Thus, overexpression of a constitutively active mutant produces effects similar to sustained activation, since the mutant cannot be down-regulated. This may result in a phenotype that is very different from that achieved by the transient activation of the same pathway that is typical of many physiological effectors.

We previously employed a combined approach, including the use of a dominant negative form of Ras (N17Ras), Ras farnesyltransferase inhibitor (PD98059), c-Raf inhibitors, and a Ras-Raf-independent pathway, including the use of a constitutively active mutant of c-Raf (RafCAAAX). H4IIE, rat hepatoma cells were transiently transfected with an expression plasmid consisting of nucleotides 467 to 1699 of the PEPCK promoter fused to a CAT reporter gene (pPL32), in combination with a control plasmid (neo), an active Ras expression plasmid (V61Ras), a glucocorticoid receptor expression plasmid (GR), a PKA catalytic subunit expression plasmid or an active c-Raf expression plasmid (RafCAAAX). A, H4IIE cells were transfected with pPL32 (15 ng) or pPL32 plus V61Ras (1 ng, Ras-active) in the presence of serum-free DMEM (con), dexamethasone (Dex, 100 nM), or Dex plus insulin (10 nM). In addition, an expression plasmid encoding the glucocorticoid receptor (GR, to enhance the dexamethasone induction) or the catalytic subunit of PKA to mimic cAMP/glucagon induction of the PEPCK gene were included where appropriate. The amount of DNA transfected in each case was normalized with RSV-neo. Cells were harvested after 24 h, and CAT activity was measured. Results are presented as an average of four experiments ± S.E. B, H4IIE cells were transfected with pPL32 and GR plus the indicated amount of a RafCAAAX expression plasmid (raf) and incubated with serum-free DMEM, dexamethasone (Dex, 100 nM), or dexamethasone plus insulin (Ins) (10 nM). The latter two treatments were repeated in the presence or absence of 10 μM PD98059 (inhibitor of MEK activation (MEK Inh)). Cells were harvested after 24 h, and CAT activity was measured. Results are presented as an average of three experiments ± S.E.
syltransferase inhibitors, and an inhibitor of MEK to block activation of p42/p44 MAP kinase in H4IIE cells by insulin. All three approaches prevented p42/p44 MAP kinase activation but had no effect on the regulation of PEPCK gene transcription by insulin (14). We now extend these studies by presenting additional evidence that the Ras MAP kinase pathway cannot mediate the repression of PEPCK expression by insulin.

First, high level expression of a dominant negative Ras molecule inhibits insulin activation of PI 3-kinase as well as insulin activation of the Ras MAP kinase pathway. Thus the reported effects of this Ras mutant on the regulation of PEPCK gene transcription by insulin could well be due to effects on PI 3-kinase, an enzyme known to be involved in the repression of PEPCK gene transcription by insulin (13) rather than the p42/p44 MAP kinase cascade. It is possible that Ras lies upstream of PI 3-kinase in an insulin signaling cascade that leads to the PEPCK promoter; however, this seems unlikely for two reasons. Ras-dependent insulin activation of p42/p44 MAP kinase is much more sensitive to N17Ras expression than is the inhibition of PI 3-kinase (Fig. 4A). Also, inhibitors of Ras farвести that prevent the proper localization of Ras in the cell have no effect on the ability of insulin to regulate PEPCK gene transcription (14). Ras can interact with PI 3-kinase in vitro (32, 33), so the inhibitory action of N17Ras could be due to a direct interaction with PI 3-kinase in vivo. Whatever the mechanism, it appears that the inhibition of PI 3-kinase and not the inhibition of the Ras MAP kinase pathway, correlates best with the loss of insulin repression of PEPCK gene transcription in H4IIE cells seen with N17Ras expression (Fig. 2).

Second, cAMP prevents activation of p42/p44 MAP kinase by hepatocyte growth factor in isolated hepatocytes and partially antagonizes epidermal growth factor-induced p42/p44 MAP kinase activity (30). In many cell types, cAMP activates the small GTPase Rap1, and this may underlie the inhibitory action of cAMP on Ras function (34). In addition, cAMP activation of PKA leads to the phosphorylation of Raf (26). In vitro phosphorylation of Raf on Ser-621 by PKA inhibits Raf activity (35). Either or both of these mechanisms could explain how cAMP blocks p42/p44 MAP kinase induction by insulin. We show here that the induction of p42/p44 MAP kinase by insulin in H4IIE cells is completely blocked by SCPT-cAMP. Since the induction of PEPCK gene transcription by SCPT-cAMP is completely prevented by insulin, there seems to be no association between this process and the effect of insulin on p42/p44 MAP kinase.

Third, although expression of an active Raf mutant can partially mimic the action of insulin on PEPCK gene transcription, its effects are blocked by PD98059 (an inhibitor of MEK activation (36)), whereas insulin action on PEPCK expression is unaffected by this agent. Thus, insulin does not invoke a Raf MAP kinase pathway to regulate the transcription of PEPCK.

These data demonstrate the importance of employing as many distinct techniques as possible to address the role of a given signaling molecule (pathway) in specific cellular processes. Although insulin uses a PI 3-kinase-dependent, Ras MAP kinase-independent mechanism to repress PEPCK gene transcription, activation of the Ras-Raf MAP kinase pathway is sufficient to partially repress PEPCK gene transcription, and it is possible that this cascade is used by other agents to repress PEPCK expression (Fig. 5). This pathway could converge with the insulin pathway at some point below p42/p44 MAP kinase. It is of interest that other workers have found that the repression of cAMP-induced PEPCK transcription by insulin is blocked by N17Ras overexpression (18). We have shown that N17Ras overexpression can partially block activation of PI 3-kinase by insulin in H4IIE cells. However, since N17Ras expression levels were not examined in either report, and Nakajima et al. (18) could not measure PI 3-kinase activity in their system, it is not possible to be certain that PI 3-kinase was inhibited in these experiments. Also, a much shorter PEPCK promoter construct was employed previously rather than the pPL32 construct used in this report. It is possible that the insulin signaling pathway to this short promoter construct is Ras-dependent, whereas the larger, more physiologic construct (pPL32), contains multiple redundant elements that can be regulated in a Ras-independent fashion by insulin. Consistent with this concept, we demonstrate that activation of the Ras MAP kinase pathway can partially repress pPL32, whereas we have previously reported that the PEPCK promoter contains at least two distinct IRSs (see Ref. 1 for review).

The best described PEPCK IRS was not present in the construct employed by Nakajima et al (18).

The Ras MAP kinase pathway has been implicated in the regulation of immediate early gene expression through DNA elements such as the AP-1 motif and the serum response element/ternary complex factor element. However, there are no consensus AP-1 or serum response element elements within the PEPCK promoter. It is clear that all of the genes induced by cAMP are not repressed by insulin; thus, the PEPCK promoter as well as other genes regulated in a similar fashion, must contain some inherent mechanism that dictates which signal is dominant. A novel mechanism has been proposed for regulation of gene expression by insulin involving the CBP/p300 co-activator protein (18). CBP/p300 is a target for multiple signaling pathways and overexpression of part of this molecule, in a stably transfected H4IIE cell line, antagonizes insulin regulation of PEPCK gene transcription (18). The CBP/p300 protein receives an inhibitory signal after the activation of the Ras MAP kinase pathway that antagonizes the effect of cAMP on gene transcription. Although the Ras MAP kinase pathway is not required for the repression of the PEPCK gene by insulin, the data presented here does not preclude a role for CBP/p300 in the regulation of PEPCK gene transcription by insulin. However, if such a mechanism is employed, a distinct signaling pathway must lead to the regulation of CBP/p300.

The data presented in this paper provide further evidence that the Ras MAP kinase pathway is not required for the regulation of PEPCK gene transcription by insulin. Activation of this pathway does partially repress PEPCK gene transcription, so it is possible that other agents that repress PEPCK gene transcription in an insulin-independent manner utilize this pathway. This work clearly illustrates the importance of employing multiple techniques when manipulating signaling pathways and shows the usefulness of a transfection system that allows the measurement of endogenous signaling molecules during such manipulations.

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