Dominant Negative Forms of Akt (Protein Kinase B) and Atypical Protein Kinase Cα Do Not Prevent Insulin Inhibition of Phosphoenolpyruvate Carboxykinase Gene Transcription*

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Ko Kotani, Wataru Ogawa†, Yasuhisa Hino, Tadahiro Kitamura, Hikaru Ueno‡, Wataru Sano, Calum Sutherland¶, Daryl K. Granner*, and Masato Kasuga

From the Second Department of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan, the Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232-0165

Transcriptional regulation of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in hepatic gluconeogenesis, by insulin was investigated with the use of adenovirus vectors encoding various mutant signaling proteins. Insulin inhibited transcription induced by dexamethasone and cAMP of a chloramphenicol acetyltransferase (CAT) reporter gene fused with the PEPCK promoter sequence in HL1C cells stably transfected with this construct. A dominant negative mutant of phosphoinositide (PI) 3-kinase blocked insulin inhibition of transcription of the PEPCK-CAT fusion gene, whereas a constitutively active mutant of PI 3-kinase mimicked the effect of insulin. Although a constitutively active mutant of Akt (protein kinase B) inhibited PEPCK-CAT gene transcription induced by dexamethasone and cAMP, a mutant Akt (Akt-AA) in which the phosphorylation sites targeted by insulin are replaced by alanine did not affect the ability of insulin to inhibit transcription of the fusion gene. Akt-AA almost completely inhibited insulin-induced activation of both endogenous and recombinant Akt in HL1C cells. Furthermore, neither a kinase-defective mutant protein kinase Cα (PKCa), which blocked insulin-induced activation of endogenous PKCa, nor a dominant negative mutant of the small GTPase Rac prevented inhibition of PEPCK-CAT gene transcription by insulin. These data suggest that phosphoinositide 3-kinase is important for insulin-induced inhibition of PEPCK gene transcription and that a downstream effector of phosphoinositide 3-kinase distinct from Akt, PKCa, and Rac may exist for mediating the effect of insulin.

The primary role of insulin is to control the plasma glucose concentration by stimulating glucose transport into muscle and adipose cells as well as by reducing glucose output from the liver (1). These actions of insulin are mediated by activation of effectors, such as glucose transporters and glycogen synthase, or by regulation of the amount of specific protein participants in metabolic pathways (1–4). Phosphoenolpyruvate carboxykinase (PEPCK),1 a rate controlling enzyme of gluconeogenesis, is one such protein whose expression is regulated by insulin (2). Insulin inhibits gluconeogenesis in the liver; thus, in the absence of this effect of insulin, as in diabetes mellitus or long term starvation, gluconeogenesis is increased (1). Transcription of the PEPCK gene is increased by various hormonal agents including glucocorticoids and glucagon (or its second messenger, cAMP), and insulin inhibits PEPCK gene transcription induced by these stimuli (2). Given that PEPCK is not known to be subject to allosteric regulation, the inhibition of gluconeogenesis by insulin in vivo is probably due to the insulin-induced decrease in the amount of PEPCK protein. Moreover, the observations that PEPCK gene expression in the liver is increased in several animal models of diabetes (5) and that transgenic animals overexpressing the PEPCK gene develop a diabetic phenotype (6, 7) also indicate the importance of this enzyme in glucose homeostasis in vivo.

Despite the recent progress in our knowledge of insulin signal transduction, the mechanism by which PEPCK gene transcription is regulated remains unclear. The Ras and mitogen-activated protein kinase signaling cascade contributes to the regulation of the expression of various genes by insulin (8). A constitutively active mutant of Ras was shown to inhibit transcription of the PEPCK gene induced by cAMP-dependent protein kinase (9). In contrast, constitutively active mutants of Ras or of Raf, an immediate downstream effector of Ras, did not inhibit PEPCK gene transcription induced by dexamethasone and a cAMP analog (10, 11). Despite the apparently discrepant results obtained with these constitutively active mutants, the observation that either a dominant negative mutant of Ras or a pharmacological inhibitor that blocks farnesylation of Ras, an important step in the activation of this GTPase, did not prevent insulin inhibition of PEPCK gene transcription (9–11) suggests that Ras is not required for this effect of insulin.

The role of PI 3-kinase, which is involved in a number of the metabolic actions of insulin (12), on PEPCK gene transcription has also been explored. Yang and Dickson (13) showed that

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1 The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; PI, phosphoinositide; PKC, protein kinase C; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin; MOI, multiplicity of infection; pfu, plaque-forming unit.

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‡ To whom correspondence should be addressed: Second Dept. of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: 81-78-382-5864; Fax: 81-78-382-2080.
Insulin-induced inhibition of PEPCK gene expression was not affected by a pharmacological blocker of PI 3-kinase, to which various metabolic actions of insulin are sensitive (12). However, a previous observation by Sutherland et al. (14), subsequently confirmed by others (10, 15), demonstrated that the inhibitory effect of insulin on PEPCK gene transcription is dependent on PI 3-kinase. The role of Akt (also known as protein kinase B), an immediate downstream effector of PI 3-kinase, in regulation of PEPCK gene transcription is also controversial. A kinase-deficient mutant of Akt, in which ligand-induced phosphorylation sites were replaced for alanine, prevents the inhibition of PEPCK transcription induced by insulin (15). By contrast, Agati et al. (9) showed that kinase-deficient Akt variants containing an amino acid substitution in the kinase domain or in the PH domain did not inhibit insulin-induced repression of PEPCK transcription.

In this study, we have further explored the role of PI 3-kinase in the inhibition of PEPCK gene transcription by insulin, by using dominant negative and constitutively active mutants of the enzyme. Moreover, we have investigated the roles of several downstream targets of PI 3-kinase, including Akt, atypical protein kinase C (PKCs), and the small GTPase Rac, in this action of insulin.

EXPERIMENTAL PROCEDURES

Cells and CAT Assay—HL1C cells, which contain the PEPCK gene promoter sequence from positions −2100 to +69 ligated to the chloramphenicol acetyltransferase (CAT) reporter gene, have been described previously (16). These cells were cultured in 60-mm plates, infected (or not) with various adenovirus vectors, and deprived of serum for 3 h before incubation for 10 h with 500 nM dexamethasone and 0.1 mM 8CPT-cAMP in the absence or presence of 100 μM insulin. The cells were then scraped into phosphate-buffered saline, separated by brief centrifugation, and resuspended in 400 μl of 250 mM Tris-HCl (pH 7.8). CAT activity in the cell extracts was then assayed as described (16).

Antibodies and Kinase Assays—The polyclonal antibodies to Akt, PKCl (α190 or α197), or PKCζ (α170) have been described (17, 18). The polyclonal antibodies to Akt recognize all three known isoforms of this protein (17). Monoclonal antibodies to the hemagglutinin (HA) epitope tag (12CA5), the FLAG epitope tag, or phosphotyrosine (PY20) were obtained from Roche Molecular Biochemicals, Kodak Scientific Imaging Systems, and Transduction Laboratories, respectively. Monoclonal antibodies to the Myc epitope tag were purified from medium supernatant of 9E10 hybridoma obtained from American Type Culture Collection. Polyclonal antibodies directed against Akt2 (protein kinase Bβ) or Akt3 (protein kinase Bγ) were obtained from Upstate Biotechnology, Inc. Polyclonal antibodies generated in response to a peptide corresponding to the COOH terminus of rat PKCβ2 (atypical CT) were obtained from Life Technologies, Inc.; these antibodies recognize both PKCα and PKCζ. (18). A monoclonal antibody to PKCα (αACT), induced by a glutathione S-transferase fusion protein containing amino acids 397–558 of mouse PKCα, was obtained from Transduction Laboratories.

HL1C cells were deprived of serum for 16–20 h, incubated in the absence or presence of 100 nM insulin for 5 min (for PI 3-kinase assay) or for 10 min (for the Akt assay), and then immediately frozen with liquid nitrogen. To assay Akt activity, the frozen cells were lysed and subjected to immunoprecipitation with antibodies directed against either Akt or HA, as described previously (17). The kinase activity in the resulting immunoprecipitates was assayed as described (17) with histone 2B as the substrate. To assay PKC activity, the frozen cells were lysed and subjected to immunoprecipitation with antibodies against PKCα (α197). The kinase activity in the resulting precipitates was assayed using myelin basic protein as the substrate (18). Frozen cells were lysed and subjected to immunoprecipitation with antibodies to Myc or to phosphotyrosine, and the resulting immunoprecipitates were assayed for PI 3-kinase activity as described (19).

Construction of and Infection with Adenovirus Vectors—Adenovirus vectors encoding a mutant regulatory subunit of PI 3-kinase that lacks the kinase domain for the catalytic subunit (AxCAαp85), HA-tagged wild-type Akt (AxCAAkt-WT), HA-tagged mutant Akt in which Lys182 is replaced by glutamate (AxCAAkt-K182E), HA-tagged mutant Akt in which Thr205 and Ser473 are replaced by alanine (AxCAAkt-AA), and a constitutively active mutant of PKCα that lacks the pseudosubstrate domain (AxCAΔPFD), or a dominant negative mutant of PKCζ that lacks the pseudosubstrate domain (AxCAΔζD), or a dominant negative mutant of PI 3-kinase (AxCAΔζD), or a dominant negative mutant of PKCζ that lacks the pseudosubstrate domain (AxCAΔζD), were obtained from Transduction Laboratories.

HL1C cells were deprived of adenovirus vectors at the indicated MOI (in pfu/cell) were deprived of serum and incubated for 10 h in the absence or presence of 500 nM dexamethasone and 0.1 mM 8CPT-cAMP (Dex-cAMP) in regulation of PEPCK gene transcription. Cells infected with AxCAΔζD at the indicated MOI (pfu/cell) were deprived of serum and incubated for 10 h in the absence or presence of 500 nM dexamethasone and 0.1 mM 8CPT-cAMP (Dex-cAMP), with or without 100 μM insulin. Cell extracts were then prepared and assayed for CAT activity as described under “Experimental Procedures.” CAT activity was corrected for variations in the amount of extract protein and expressed as a percentage of that obtained from cells that were exposed to dexamethasone and 8CPT-cAMP alone. The results illustrated in A are representative of three independent experiments. Data in B represent the mean ± S.E. of three experiments.

RESULTS

Role of PI 3-Kinase in the Inhibition of PEPCK Gene Transcription by Insulin—We first investigated the effect of a dominant negative mutant of PI 3-kinase (Δp85), which contains a mutant regulatory subunit that lacks the binding site for the 110-kDa catalytic subunit of the enzyme (19, 21) on insulin-induced repression of PEPCK transcription. Insulin induced an almost complete abolition of Δp85 on PEPCK transcription in HL1C cells (Fig. 1A). Infection of the cells with an adenovirus vector encoding Δp85 (AxCAΔp85) inhibited the insulin-induced increase of PI 3-kinase activity in a dose-dependent manner; at an MOI of 10 plaque-forming units (pfu) per cell, the effect of insulin was almost completely abolished (Fig. 1A). The combination of dexamethasone and 8-chlorophenylthio-cAMP (dexamethasone-cAMP) induced a 30–50-fold increase of PEPCk promoter in HL1C cells (Fig. 1B), and incubation of these cells with insulin resulted in inhibition of dexamethasone-cAMP-stimulated...
transcription from the PEPCK-CAT reporter gene by ~80–90% (Fig. 1B), which is consistent with the results of previous studies (10, 14, 16). The stimulation of CAT activity by dexamethasone-cAMP in cells infected with AxCAΔp85 was similar to that noted in noninfected cells (Fig. 1B), indicating that Δp85 did not affect the induction of PEPCK gene transcription by dexamethasone-cAMP. However, infection with AxCAΔp85 resulted in a dose-dependent increase in transcription of the PEPCK-CAT reporter gene in cells incubated in the presence of dexamethasone-cAMP and insulin. At an MOI of 10 pfu/cell, a virus dose sufficient to almost completely inhibit insulin activation of PI 3-kinase, inhibition of PEPCK gene transcription by insulin was completely prevented.

To further investigate the role of PI 3-kinase in the regulation of PEPCK gene transcription, we examined the effect of a constitutively active mutant of this enzyme. Myr-p110, a chimeric protein consisting of the catalytic subunit of PI 3-kinase ligated to a myristoylation signal sequence at its NH₂ terminus and with the Myc epitope at its COOH terminus, was expressed in HL1C cells with the use of an adenovirus vector (AxCAMyr-p110). Expression of similar forms of p110 in quiescent cells both stimulates the production of D3-phosphorylated phosphoinositides and enhances various biological activities located downstream of PI 3-kinase (22). Infection of HL1C cells with AxCAMyr-p110 resulted in a dose-dependent increase both in the amount of the chimeric protein and in the activity of PI 3-kinase (Fig. 2, A and B). Infection of HL1C cells with AxCAMyr-p110 also resulted in a dose-dependent inhibition of the dexamethasone-cAMP-mediated expression of the PEPCK-CAT reporter gene (Fig. 2C). These results with Δp85 and Myr-p110 suggest that PI 3-kinase is required for insulin-induced inhibition of PEPCK gene transcription.

Role of Akt in the Regulation of PEPCK Gene Transcription—We next examined the role of Akt, a downstream effector of PI 3-kinase, in the regulation of PEPCK gene transcription. We first tested the effect of a constitutively active mutant of Akt. The kinase activity of Myr-Akt, which consists of Akt1 ligated to a myristoylation sequence, is ~10 times greater than that of the wild-type enzyme. The expression of this mutant in quiescent 3T3-L1 adipocytes stimulates glucose transport (data not shown), which is consistent with the results reported in a previous study (23). Infection of HL1C cells with a vector that expresses AxCAMyr-Akt resulted in a dose-dependent inhibition of dexamethasone-cAMP-induced CAT activity (Fig. 3), which suggests that signaling through Akt results in inhibition of PEPCK gene transcription.

We have previously shown that a mutant Akt, in which the sites of ligand-induced phosphorylation are replaced by alanine (Akt-AA), is not activated by insulin. Akt-AA exerts dominant inhibitory effects on insulin-induced activation of endogenous or transfected Akt in 3T3-L1 adipocytes or Chinese hamster ovary cells (17). HL1C cells were infected with an adenovirus vector that encodes HA-tagged wild-type Akt1 (AxCAAkt-WT) at an MOI of 0.5 pfu/cell and, 12 h later, with an adenovirus encoding FLAG-tagged Akt-AA (AxCAAkt-AAFL) at various doses. The cells were subsequently incubated in the absence or presence of insulin, lysed, and subjected to immunoprecipitation with antibodies to HA. An assay of the resulting immunoprecipitates for Akt1 activity revealed that infection of the cells with AxCAAkt-AAFL inhibited insulin-induced activation of Akt-WT in an MOI-dependent manner (Fig. 4B) without affecting the amount of Akt-WT protein (Fig. 4A). At an MOI of 10 pfu/cell, insulin-induced activation of Akt-WT was almost completely abolished.

We next investigated the effect of Akt-AA on endogenous Akt activity in HL1C cells by precipitating the endogenous protein with polyclonal antibodies to Akt. Because these antibodies recognize recombinant Akt proteins, Akt-AA was immunodepleted from cell lysates with antibodies to FLAG before the

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**Fig. 2.** Effect of Myr-p110 on dexamethasone-cAMP-induced PEPCK gene transcription in HL1C cells. A and B, expression and activity of Myc-tagged myristoylated p110 (Myr-p110). Cells were infected with AxCAMyr-p110 at the indicated MOI (pfu/cell). After 48 h, cell lysates were prepared and subjected to immunoblot analysis (A) or to immunoprecipitation (B) with antibodies directed against Myc. The immunoprecipitates were assayed for PI 3-kinase activity. The results shown are representative of three independent experiments. C, effect of Myr-p110 on PEPCK gene transcription. Cells infected with AxCAMyr-p110 at the indicated MOI (pfu/cell) were deprived of serum, incubated in the absence or presence of dexamethasone-cAMP, and then assayed for CAT activity. Data represent the mean ± S.E. of three experiments.

**Fig. 3.** Effect of a constitutively active mutant of Akt on dexamethasone-cAMP-induced PEPCK gene transcription. HL1C cells infected with AxCAMyr-Akt at the indicated MOI (pfu/cell) were deprived of serum and then incubated in the absence or presence of dexamethasone-cAMP and insulin. CAT activity was assayed as described above. The data represent the mean ± S.E. of three experiments.

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endogenous Akt was immunoprecipitated with the polyclonal antibodies and assayed for kinase activity. Infection of HL1C cells with AxCAAkt-AAFL resulted in an MOI-dependent increase of the amount of total Akt protein (Fig. 4C); at an MOI of 10 pfu/cell, the abundance of Akt-AA was ∼20 times that of endogenous Akt (data not shown). After three sequential immunoprecipitations with antibodies to FLAG, the amount of Akt protein remaining in the supernatant was similar for infected and noninfected cells (Fig. 4C), indicating that Akt-AA was quantitatively removed by this procedure. The insulin-induced activation of endogenous Akt was inhibited by AxCAAkt-AAFL in an MOI-dependent manner; the inhibition was >90% at an MOI of 10 pfu/cell (Fig. 4D). These results suggest that Akt-AA exerts a dominant negative effect on the activation of both endogenous and transfected Akt by insulin in HL1C cells. The dominant negative effect of Akt-AA was confirmed by an assay of endogenous Akt2 activity precipitated with antibodies to Akt2. Because Akt-AA was constructed from Akt1, the polyclonal antibodies to Akt2 did not recognize the mutant protein (data not shown). Insulin induced an ∼2.5-fold increase in endogenous Akt2 activity (Fig. 5A). Infection of cells with AxCAAkt-AA, which encodes HA-tagged Akt-AA, at an MOI of 10 pfu/cell inhibited the insulin-induced increase in Akt2 activity by more than 90% (Fig. 5A). Polyclonal antibodies against Akt3 did not precipitate significant Akt activity over a control precipitation from HL1C cells (data not shown).

With the use of Akt-AA, we then examined the effect of inhibition of insulin-induced Akt activation on PEPCK gene transcription. Infection of HL1C cells with AxCAAkt-AA did not affect the increase in the expression of the PEPCK-CAT reporter gene induced by dexamethasone-cAMP (Fig. 5B). Furthermore, the repression of CAT activity by insulin was also not affected by infection of HL1C cells with AxCAAkt-AA. Infection with AxCAAkt-AAFL also had no effect on transcription of the PEPCK-CAT reporter gene in HL1C cells (data not shown).

A kinase-deficient mutant of Akt, in which Lys179 in the kinase domain is replaced by neutrally charged amino acids, exerts dominant negative effects on various insulin-induced biological activities, including the inhibition of insulin-like growth factor-binding protein-1 gene transcription (24), although this and similar kinase-defective mutants of Akt do not inhibit insulin-induced activation of Akt (17, 25). We therefore tested for an effect on PEPCk gene transcription of an adenovirus that encodes a mutant Akt, in which the Lys179 in the kinase domain is replaced by aspartate (AxCAAkt-K179D). HL1C cells were infected with adenovirus encoding AxCAAkt-K179D. HL1C cells were infected with adenovirus encoding AxCAAkt-K179D, the cells were then incubated in the absence or presence of insulin, after which Akt activity was assayed following precipitation with polyclonal antibodies directed against Akt. Although the amount of Akt-K179D protein in the cells infected with AxCAAkt-K179D at an MOI of 10 was similar to that in cells infected with AxCAAkt-AA at a given MOI (data not shown), the activation of endogenous Akt2 by insulin was not inhibited by Akt-K179D (Fig. 5C). This suggests that Akt-K179D does not prevent the activation of endogenous Akt.

Roles of Atypical Isoforms of PKC in Regulation of PEPCK
Insulin Regulation of PEPCK Gene Transcription—We have recently shown that PKCα is activated by insulin through a PI 3-kinase-dependent mechanism and that this isoform contributes to the stimulation of glucose transport by insulin in 3T3-L1 adipocytes (18). We therefore investigated whether atypical isoforms of PKC participate in the regulation of PEPCK gene transcription by insulin. We first examined whether either of the atypical isoforms of PKC, PKCα or PKCζ, is expressed in HL1C cells. Both HL1C and 293 cells were lysed and subjected to immunoprecipitation with antibodies against PKCα (α190) or PKCζ (α170), and the resulting precipitates were then subjected to immunoblot analysis with antibodies that recognize both PKCα and PKCζ (atypical CT). The α190 and α170 antibodies specifically recognize PKCα and PKCζ, respectively (18). Both α190 and α170 precipitated proteins of ~80 kDa from 293 cells that reacted with atypical CT on immunoblot analysis (Fig. 6A), suggesting that both of the isoforms are expressed in these cells. In contrast, α190, but not α170, precipitated an ~80-kDa protein from HL1C cells that was recognized by atypical CT, suggesting that only PKCα is expressed in these cells.

Exposure of HL1C cells to insulin resulted in a ~1.5-fold increase in the amount of kinase activity that was precipitated with antibodies to PKCα (α197) (Fig. 6C). Infection of the cells with an adenovirus encoding a dominant negative mutant of PKCα (AxCAÅNKD) (18) resulted in a dose-dependent increase in the amount of the mutant protein (Fig. 6D) as well as an MOI-dependent inhibition of the insulin-induced increase in PKCα activity (Fig. 6C); at an MOI of 10 pfu/cell, this effect of insulin was almost completely abolished. However, infection of HL1C cells with AxCAÅNKD, even at an MOI of 10 pfu/cell, did not affect the inhibition of transcription of the PEPCK-CAT reporter gene by insulin (Fig. 6D). Furthermore, expression of λPD, a constitutively active mutant of PKCα, which is capable of stimulating glucose transport in quiescent 3T3-L1 cells (18), did not mimic the inhibitory effect of insulin on PEPCK gene transcription (Fig. 6E). These results suggest that PKCα does not contribute to this effect of insulin.

The Role of Rac in the Regulation of PEPCK Gene Transcription—Given that the small GTPase Rac has been implicated in signaling downstream of PI 3-kinase (12), we examined the effect of a dominant negative mutant of this protein on PEPCK gene transcription. Infection of HL1C cells with AxCAŔac17N resulted in a dose-dependent increase in the amount of the mutant protein (Fig. 7A), but it had no effect either on the increase in PEPCK gene expression induced by dexamethasone-cAMP or on the inhibition of this effect by insulin (Fig. 7B). This result suggests that signals transmitted through Rac do not contribute to the regulation of PEPCK gene transcription by insulin.

DISCUSSION

Pharmacological inhibitors of PI 3-kinase, such as wortmannin and LY294002, are useful tools for exploring insulin signal transduction. Various metabolic actions of insulin, including stimulation of glucose transport, glycogen synthase, amino acid transport, and general protein synthesis, are sensitive to these inhibitors (3, 4, 12). These compounds also prevent the represion of PEPCK gene transcription by insulin (10, 14, 15), but like most inhibitors, their effects may not be completely specific, and a number of approaches should be used in assessing the role of a particular signal transduction step in a given pathway. Accordingly, we here show that a dominant negative

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mutant of PI 3-kinase, Dp85, blocked this effect of insulin. Moreover, a constitutively active form of PI 3-kinase (Myr-p110) mimicked the effect of insulin on PEPCK gene expression. These data indicate that insulin-induced inhibition of PEPCK gene transcription is mediated by PI 3-kinase. Akt variants in which the Akt sequence is ligated to either a myristoylation signal sequence or a viral Gag protein exhibit kinase activity that is greater than that of the wild-type enzyme (26, 27). The expression of such mutants promotes glucose transport, general protein synthesis, glycogen synthase activity, p70 S6 kinase activity, and phosphorylation of 4E-BP1 (also known as PHAS1) (26–28), all of which are stimulated by insulin in a PI 3-kinase-dependent manner (12). We have now shown that stimulation of PEPCK gene transcription by dexamethasone-cAMP was markedly attenuated in HL1C cells that express a constitutively active mutant of Akt (Myr-Akt), an observation consistent with the results of a previous study (15). However, whereas the expression of Akt-AA inhibited the insulin-induced activation of Akt as well as insulin-induced stimulation of glycogen synthase (29), phosphorylation of PHAS1 (29), and phosphorylation and activation of the 3β isofrom of cAMP phospho-

FIG. 6. Effect of a dominant negative mutant of PKC (ΔNKD) on insulin-induced activation of PKC activity and insulin inhibition of PEPCK gene transcription. A, expression of endogenous atypical PKC in HL1C and 293 cells. Cells were lysed and subjected to immunoprecipitation (IP) with α190 or with α170, and the resultant precipitates were subjected to immunoblot analysis with atypical CT. The 80-kDa region of the blots is shown. B and C, expression of ΔNKD and its effect on insulin-induced activation of endogenous PKCA activity. HL1C cells were infected with or without AxCAΔNKD at the indicated MOI. After 48 h, the cells were treated with or without insulin and lysed, and the lysates were subjected to immunoprecipitation analysis with atypical CT (B) or to immunoprecipitation with α197 (C). The immunoprecipitates were then assayed for PKCA activity as described under “Experimental Procedures.” D, effect of ΔNKD on insulin-induced suppression of PEPCK transcription. HL1C cells infected with or without AxCAΔNKD were deprived of serum for 3 h and incubated with or without dexamethasone and 8CPT-cAMP (Dex/cAMP) in the presence or absence of insulin for 10 h, following which CAT activity was assessed. E, effect of a constitutively active mutant of PKC (ΔPD) on dexamethasone-cAMP-induced PEPCK gene transcription. HL1C cells infected with AxCAΔPD at the indicated MOI (pfu/cell) were deprived of serum and then incubated in the absence or presence of dexamethasone-cAMP and insulin. CAT activity was assayed as described above. Data in C–E represent the means ± S.E. of three experiments.

FIG. 7. Effect of a dominant negative mutant of Rac (Rac17N) on insulin-induced suppression of PEPCK gene transcription. A, HL1C cells were infected with or without AxCARac17N at the indicated MOI. After 48 h, the cells were lysed, and the lysates were subjected to immunoblot analysis with antibodies directed against HA (12CA5). B, HL1C cells infected with or without AxCARac17N at the indicated MOI were deprived of serum for 3 h and incubated with or without dexamethasone and 8CPT-cAMP (Dex/cAMP) in the presence or absence of insulin for 10 h, following which CAT activity was assessed. Data in B represent the means ± S.E. of three experiments.
diesterase (PDE3B), this mutant did not affect the inhibition of PEPCK gene transcription by insulin.

It is possible that Akt-AA did not completely prevent signal transmission through endogenous Akt and that the remaining low level of Akt activity was sufficient to inhibit PEPCK gene transcription to a substantial extent. However, this possibility is unlikely because both insulin-induced Akt activation (data not shown) and PEPCK gene transcription were inhibited by a dominant negative mutant of PI 3-kinase (Δp85) in a similar dose-dependent manner, suggesting that a small increase in the activity of Akt is not sufficient to fully inhibit PEPCK gene expression. Therefore, the simplest explanation of the present results is that a molecule distinct from Akt is capable of transmitting signals to PEPCK gene transcription, whereas activated Akt is sufficient to inhibit the expression of this gene under certain conditions.

We have shown that Akt-AA almost completely abolished the stimulation of transected Akt1 and endogenous Akt2 activity by insulin. Because antibodies against Akt3 did not precipitate insulin-stimulated kinase activity from H1C cells, this isoform of Akt may not be expressed in these cells. Furthermore, we have shown that Akt-AA inhibited insulin-induced endogenous Akt activity precipitated with antibodies that recognize all three known isoforms of Akt with apparent similar efficiency (17). However, Akt translocates to the plasma membrane fraction or GLUT4-containing vesicles in response to extracellular stimuli (30, 31), suggesting that translocation of Akt to a specific intracellular compartment is important for its activation and signal transmission. Because we assayed Akt activity in immunoprecipitates prepared from total cell lysates, it is possible that this assay does not completely reflect the activity of Akt in a specific cellular compartment. We therefore cannot exclude the possibility that an increase of Akt activity in a certain fraction of the cell may be sufficient to transmit signals to the PEPCK gene promoter. It is also possible that an unidentified isoform of Akt that is resistant to Akt-AA is present in the cells studied and responsible for the regulation of PEPCK gene transcription.

Liao et al. (15) showed that insulin-induced inhibition of PEPCK gene transcription is partially blocked in H4IE cells that stably express Akt-AA (15). However, these investigators did not observe the inhibition of insulin-induced activation of endogenous Akt by this mutant. The reason for this apparent discrepancy between their results and ours is not clear. The difference could be related to the methods used to monitor PEPCK mRNA expression. Whereas we assayed transcription by assessing the activity of a CAT reporter gene fused to the promoter region of the PEPCK gene, Liao et al. (15) measured PEPCK mRNA by primer extension analysis. The latter technique cannot discriminate between effects on gene transcription and mRNA stability, and insulin is known to affect PEPCK mRNA stability (2).

We and others have shown that a kinase-defective mutant of Akt in which the Lys179 in the kinase domain is replaced by aspartate does not inhibit insulin-induced Akt activation (17, 25). Nonetheless, this and similar Akt mutants do inhibit certain biological actions of insulin, including the phosphorylation of 4E-BP1 (32), the activation of glycogen synthase (29), and phosphorylation and activation of PDE3B. Because Bel-XI/
Bcl-2-associated death factor and glycogen synthase kinase-3β, putative in vivo substrates of Akt, associate with Akt in intact cells (25, 33), it is possible that kinase-defective mutants of Akt containing substitutions at Lys179 block signaling downstream of Akt by competing with the endogenous enzyme for its substrates. However, in the present study, infection of HL1C cells with AxCAAkt-K179D did not prevent the inhibition of PEPCK gene transcription by insulin, which is consistent with the results of a previous study (9). These observations also support the hypothesis that inhibition of Akt signaling is not sufficient to prevent insulin suppression of PEPCK gene transcription.

Atypical isoforms of PKC are also thought to act downstream of PI 3-kinase (18, 34, 35). We have previously shown that expression of ΔλNKD markedly inhibits stimulation of both PKCα activity and glucose transport in 3T3-L1 adipocytes by insulin (18). We have now shown that PKCλ, but not PKCe, is expressed in HL1C cells and that ΔλNKD does not affect the inhibition of PEPCK gene transcription by insulin, although this mutant almost completely inhibited insulin-induced activation of PKCα. Atypical PKC has been proposed to participate in the regulation of the mitogen-activated protein kinase-signaling cascade (36, 37). In this regard, we have recently shown that expression of ΔλNKD inhibits the insulin-induced activation of mitogen-activated protein kinase in various cells, including HL1C cells. These results, together with our observation that ΔλPD, a constitutively active mutant of PKCα, does not mimic the effect of insulin on PEPCK gene transcription, indicate that PKCα does not participate in this effect of insulin.

The small GTPase Rac, another downstream effector of PI 3-kinase, mediates insulin-induced formation of lamellipodia (38). Overexpression of a dominant negative mutant of Rac (Rac17N) did not prevent the inhibitory effect of insulin on PEPCK gene transcription, suggesting that the Rac pathway does not contribute to this action of insulin. Because insulin does not induce formation of lamellipodia in HL1C cells, we do not know whether the level of expression of Rac17N achieved in the present study is sufficient to prevent signaling through endogenous Rac. However, given that insulin-induced formation of lamellipodia is completely blocked in Chinese hamster ovary cells, KB cells, and 3T3-L1 adipocytes by expression of Rac17N at levels similar to or lower than that achieved in the present study (data not shown), it is likely that the concentration of Rac17N achieved in HL1C cells was sufficient to inhibit the endogenous protein.

In summary, we have shown that a constitutively active mutant of PI 3-kinase inhibits the dexamethasone-CAMP-induced stimulation of PEPCK gene transcription and that a dominant negative mutant of this lipid kinase blocks insulin-induced inhibition of PEPCK gene expression. However, dominant negative mutants of Akt, PKCα, and Rac did not affect the ability of insulin to inhibit transcription of the PEPCK gene. Our data suggest that a downstream effector of PI 3-kinase distinct from Akt, PKCα, and Rac mediates the effect of insulin on PEPCK gene transcription. Given that various additional enzymes, including PKCε (39), p21-activated kinase (40), and integrin-linked kinase (41), have been shown to act downstream of PI 3-kinase in insulin-induced or other growth factor-induced signaling, the effects of specific inhibition of such effectors on PEPCK gene transcription warrant investigation.

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