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New connections in the regulation of PEPCK gene expression by insulin

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SUMMARY

Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the rate-limiting step in hepatic gluconeogenesis. Glucagon (via the second messenger cAMP) and glucocorticoids stimulate transcription of the PEPCK gene whereas insulin and phorbol esters have a dominant inhibitory effect. Wortmannin, an inhibitor of 1-phosphatidylinositol 3-kinase (PI 3-kinase), blocks the inhibition of glucocorticoid- and cAMP-stimulated PEPCK gene transcription by insulin. By contrast, although phorbol esters mimic the action of insulin on the regulation of PEPCK gene transcription, wortmannin does not block the effect of these agents. Thus PI 3-kinase is required for the regulation of PEPCK gene expression by insulin but not by phorbol esters. In liver cells, insulin administration stimulates the activity of multiple protein kinases, including the p42/p44 Mitogen Activated Protein (MAP) kinase and the p70/p85 ribosomal protein S6 kinase. Selective inhibition of the activation of either kinase, utilizing the compounds PD98059 and rapamycin respectively, does not affect insulin regulation of PEPCK gene transcription. Thus regulation of PEPCK gene transcription requires PI 3-kinase but does not require the activation of either p42/p44 MAP kinase or p70/p85 ribosomal protein S6 kinase.

1. INTRODUCTION

Diabetes Mellitus is the third most prevalent disease in the western world (after heart disease and cancer), constitutes a major risk factor for heart disease and is classified as either insulin-dependent (IDDM) or non-insulin-dependent (NIDDM), the latter accounting for about 90% of recorded cases. IDDM is characterized by an inability to produce insulin and can be treated by daily injections of the hormone. In contrast, patients with fully developed NIDDM have a defect in insulin secretion (from the β cells of the pancreatic islets) and exhibit a reduced metabolic response to this hormone (insulin resistance) (Defronzo 1988; Granner & O'Brien 1992). Whether a defect in insulin secretion or insulin resistance constitutes the primary lesion in the etiology of NIDDM is the subject of continued debate. Several potential mechanisms to explain the cause of insulin resistance have been postulated (see Kahn 1994 for review) but to identify potential therapeutic targets for this syndrome, the molecular mechanisms that underlie insulin action must first be elucidated. Insulin elicits a variety of metabolic changes in many mammalian tissues, primarily adipose, liver and muscle (Denton 1986). Alterations in the activity of a multitude of proteins occurs within minutes of insulin administration to these tissues (Myers *et al.* 1994*a*; White & Kahn 1994). Similarly, by regulating multiple steps in the flow of information from gene to protein, insulin can affect the amount of over 100 proteins (for a review see O'Brien & Granner 1995). Gene transcription, mRNA stability and mRNA translation have all been identified as major targets in the regulation of gene expression by insulin (O'Brien & Granner 1995). In an attempt to map the signalling pathway(s) that lie between the specific cell membrane receptor for insulin and the promoters of insulin responsive genes, several researchers have characterized the insulin receptor along with many of its substrates/binding proteins (Cheatham & Kahn 1995). This approach has identified many potential signalling molecules that can be activated by the insulin receptor tyrosine kinase (see below). Our laboratory has undertaken a potentially more specific approach. We have begun to identify signalling molecules absolutely required for the regulation of one well-characterized insulin responsive gene, while at the same time trying to discount insulin signalling molecules not required for this regulation.

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2. INSULIN SIGNALLING AND REGULATION OF PEPCK GENE EXPRESSION

Although, to date, insulin has been shown to regulate the expression of ~100 genes (O'Brien & Granner 1995), the regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression has been studied in the most detail. PEPCK catalyses the rate-limiting step in hepatic gluconeogenesis and expression of the cytoplasmic form (but not the mitochondrial form) of the PEPCK gene is under hormonal regulation (O'Brien & Granner 1990; Hanson & Patel 1994).

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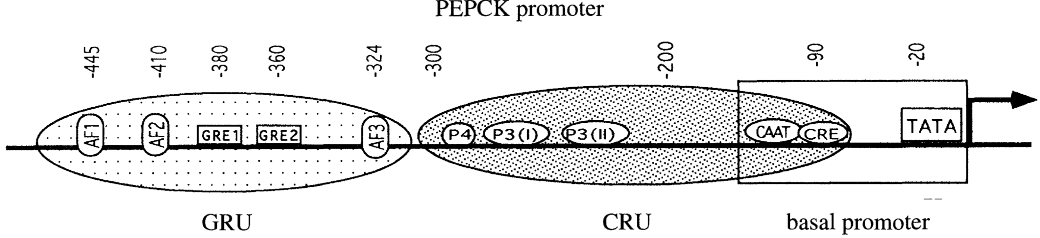

Figure 1. Structure of the PEPCK gene promoter. Cis-acting elements, identified to date, involved in the hormonal regulation of PEPCK gene transcription. The numbers identify nucleotide position relative to the transcription initiation site. In the GRU the distances in nucleotides from the transcription initiation site are measured to the centre of each element. Abbreviations are as follows. GRU glucocorticoid response unit; GRE glucocorticoid response element; AF accessory factor; CRU cAMP response unit; CRE cAMP response element.

There is no evidence for the regulation of the activity of pre-existing PEPCK molecules (for example, by post-translational modification or allosteric activation), therefore PEPCK activity is regulated entirely at the level of gene expression. In rat liver, or in the rat hepatoma cell line H4IIE, glucagon (via cAMP), glucocorticoids, thyroid hormone and retinoic acid all stimulate transcription of the PEPCK gene (O'Brien & Granner 1995). In contrast, insulin and phorbol esters inhibit basal expression of the PEPCK gene as well as reducing, in a dominant fashion, cAMP/glucocorticoid stimulation of this gene (O'Brien & Granner 1991). Many of the positive cis-acting elements within the PEPCK promoter have been identified through the analysis of a variety of PEPCK-CAT fusion genes in transient transfection experiments (see figure 1 and also O'Brien & Granner 1995). A complex glucocorticoid response unit (GRU) mediates the stimulatory action of glucocorticoids (Imai *et al.* 1990). This GRU consists of three accessory factor binding sites (AF1, from −455 to −431; AF2, from −420 to −403; AF3, from −337 to −312) and two glucocorticoid receptor binding sites, also termed glucocorticoid response elements (GRE1 and GRE2, from −395 to −349). The accessory factors do not function as glucocorticoid response elements themselves. However, if any one of these elements is mutated the PEPCK promoter loses approximately 50% of its ability to respond to glucocorticoids. Similarly, when two out of three of the elements are mutated the promoter is unresponsive to glucocorticoids demonstrating that GRE1 and GRE2 are inert by themselves, at least in the context of the PEPCK promoter (Imai *et al.* 1990; Scott & Granner, unpublished data). In addition, at least four cis-acting elements (designated the CRE, P3[I], P3[II] and P4) are required for cAMP-stimulated PEPCK gene transcription (Hanson & Patel 1994). Together these elements form a cAMP response unit. The CRE may also be required for a complete ability to respond to glucocorticoids and may thus also form part of the GRU (Imai *et al.* 1993; Angrand *et al.* 1994).

The identification of multiple elements required for the dominant negative effect of insulin was achieved using both transient and stable transfection techniques (O'Brien & Granner 1995). At least one such insulin response sequence (IRS) resides between −271 and +69 relative to the transcription start site of the PEPCK promoter, however its precise location is unclear. Another, located between −413 and −407 has been well characterized both in the context of the PEPCK promoter, and also by analysing this element in a heterologous context (O'Brien *et al.* 1995). This sequence is located within the AF2 binding region (see figure 1), and mediates both an insulin and phorbol ester signal to the PEPCK promoter (O'Brien *et al.* 1991). It has the core sequence T(G/A)TTTG (O'Brien *et al.* 1995), and a number of proteins, including C/EBP α and HNF-3 α and β bind to this IRS *in vitro* (O'Brien *et al.* 1994*a*, 1995). However, it remains unclear which, if any, of these DNA binding proteins actually mediates the action of insulin on PEPCK gene transcription.

One of the most intensely studied aspects of insulin action is how the insulin receptor transmits a signal from the plasma membrane to a *trans*-acting factor that binds specifically to a *cis*-acting IRS within the promoter of certain genes (Denton 1986; White & Kahn 1994; O'Brien & Granner 1995). The insulin receptor, as well as a number of enzymes whose activity is altered by insulin have been well-characterized (Avruch *et al.* 1994; Myers *et al.* 1994*a*; White & Kahn 1994). Unfortunately, determining the steps by which insulin can produce alterations in the activities of specific enzymes has proved difficult (see Exton 1991; Denton & Tavare 1995, and references within).

We have utilized the HL1C cell line to map the signal transduction pathway(s) involved in insulin- and phorbol ester-regulated PEPCK gene expression. HL1C cells were generated by stably transfecting a PEPCK-CAT fusion gene into the H4IIE rat hepatoma cells (Forest *et al.* 1990). This sequence (from −2100 to +69, relative to the transcription start site of the PEPCK gene), contains all of the elements described above. Individually, dexamethasone (three to fivefold) and cAMP (about 1.2-fold) stimulate CAT expression in these cells, while together they act synergistically to promote a 20- to 50-fold increase in CAT expression. Insulin acts, in a dominant fashion, to block these stimulatory effects (see figure 2; and also Forest *et al.* 1990; O'Brien *et al.* 1990, 1991, 1994*b*).

Phorbol esters, which are known to activate certain members of the protein kinase C (PKC) family, mimic the action of insulin on several genes. For example, phorbol esters and insulin induce a similar increase in c-fos mRNA levels in 3T3-L1 adipocytes (Stumpo &

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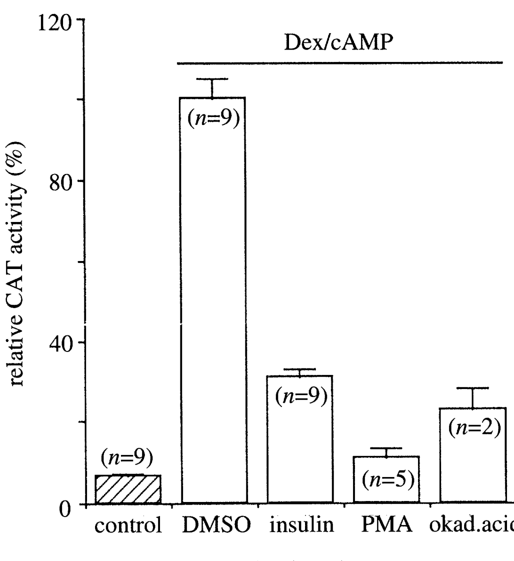

Figure 2. Stimulation of PEPCK-CAT fusion gene expression by dexamethasone and cAMP is inhibited by insulin, phorbol ester and okadaic acid. HL1C cells were incubated in serum-free medium containing 500 nM dexamethasone and 0.1 mM 8CPT-cAMP (Dex/cAMP) along with 10 nM insulin (Insulin), 1μM PMA (PMA), 10μM okadaic acid (Okad. acid), or 0.05% DMSO (DMSO). As a control, cells were incubated with 0.05% DMSO only (control). After 3 h at 37 °C the cells were harvested and assayed for CAT activity as described previously (Sutherland *et al.* 1995). Results are expressed as % CAT activity relative to Dex/cAMP stimulated activity. Results represent the mean ± standard error of the number of experiments shown in parenthesis.

Blackshear 1986) and H4IIE hepatoma cells (Messina *et al.* 1992). Similarly, phorbol esters inhibit both cAMP- and glucocorticoid-stimulated tyrosine aminotransferase gene transcription (Messina & Weinstock 1994) and like insulin, inhibit both cAMP- and glucocorticoid-stimulated PEPCK gene transcription (see figure 2, and see O'Brien & Granner 1991). However, prolonged treatment of various cell lines with phorbol esters abolishes the inhibitory effect of subsequent phorbol ester treatment on both PEPCK and c-fos gene expression, whereas the effect of insulin is unaltered (Stumpo & Blackshear 1986; Chu & Granner 1987; Taub *et al.* 1987). This suggests the existence of distinct signalling pathways for insulin and phorbol esters. However, the initially distinct insulin and phorbol ester signals (Chu *et al.* 1987) converge on the same elements within the respective gene promoters of PEPCK and c-fos, although the IRS's within the PEPCK and c-fos promoters constitute very different sequences (Stumpo *et al.* 1988; O'Brien *et al.* 1990, 1991).

Okadaic acid, a potent inhibitor of the serine/threonine protein phosphatases, types 1 and 2A (Cohen *et al.* 1990), also mimics the effect of insulin on PEPCK gene expression (see figure 2 and also O'Brien *et al.* 1994*b*). Although it has been known for some time that activation of the intrinsic tyrosine kinase activity of the insulin receptor mediates a very early, and probably essential, event in insulin signalling, this effect of okadaic acid implies a role for serine/threonine phosphorylation in the regulation of PEPCK gene expression by insulin. Thus we have examined the potential roles of a number of known insulin regulated serine/threonine protein kinases in the regulation of PEPCK gene expression by insulin and phorbol esters.

2. INSULIN SIGNALLING AND MITOGEN ACTIVATED PROTEIN KINASE

The identification of insulin receptor substrate-1 (IRS-1), as a major physiological substrate for the insulin receptor tyrosine kinase, has greatly improved our understanding of insulin signalling (for reviews see Myers *et al.* 1994*b*; White & Kahn 1994; Denton & Tavare 1995). This, along with investigations into signalling from a variety of growth factor receptors, has led to the elucidation of a multicomponent pathway beginning with tyrosine-phosphorylated IRS-1 binding growth factor receptor binding protein-2 (GRB-2) via its src homology domain-2 (SH2) (Denton 1986; Myers *et al.* 1994*b*; White & Kahn 1994; Denton & Tavare 1995). GRB-2 acts as an adaptor molecule linking, through its SH3 domain, the guanine nucleotide exchange factor mSOS. mSOS stimulates the production of the active, GTP bound form of $p21^{ras}$ which in turn causes the activation of a cascade of protein kinases (see figure 3). Firstly GTP:$p21^{ras}$ interacts with and aids in the stimulation of the serine/threonine protein kinase Raf-1. Raf-1 phosphorylates and activates the dual specificity protein kinase MEK, which can then phosphorylate (on both tyrosine and threonine residues) and activate the p42/p44 isoforms of the Mitogen Activated Protein (MAP) kinase family of protein kinases (Ahn *et al.* 1991; Gomez & Cohen 1991). Cohen *et al.* have elucidated the mechanisms whereby activation of the MAP kinase cascade may be involved in the stimulation of glycogen synthesis by insulin in rabbit skeletal muscle (Dent *et al.* 1990; Sutherland *et al.* 1993; Sutherland & Cohen 1994). In addition, the recent isolation of an inhibitor (PD98059) of MEK, (Dudley *et al.* 1995), has allowed the examination of the role of p42/p44 MAP kinase in a number of insulin-regulated processes. This inhibitor does not affect insulin-stimulated glucose uptake, lipogenesis or glycogen synthesis in 3T3-L1 adipocytes or L6 myotubes, but does block the stimulation of MAP kinase and $p90^{rsk}$ by insulin (Lazar *et al.* 1995). Thus the role of p42/p44 MAP kinase in the regulation of glycogen synthesis has been brought into question. However, because MAP kinase translocates to the nucleus following stimulation of cells with serum or growth factors (Chen *et al.* 1992; Traverse *et al.* 1992; Gonzalez *et al.* 1993), it may influence gene transcription directly through transcription factor phosphorylation or indirectly by influencing the phosphorylation of other nuclear protein kinases (for example, $p90^{rsk}$ (Sturgill *et al.* 1988), MAPKAP kinase-2 (Stokoe *et al.* 1992) and GSK-3 (Sutherland *et al.* 1993; Sutherland & Cohen 1994). It is not yet known whether MAP kinase can translocate to the nucleus of liver cells after stimulation by insulin, however, PD98059 blocks the transcrip-

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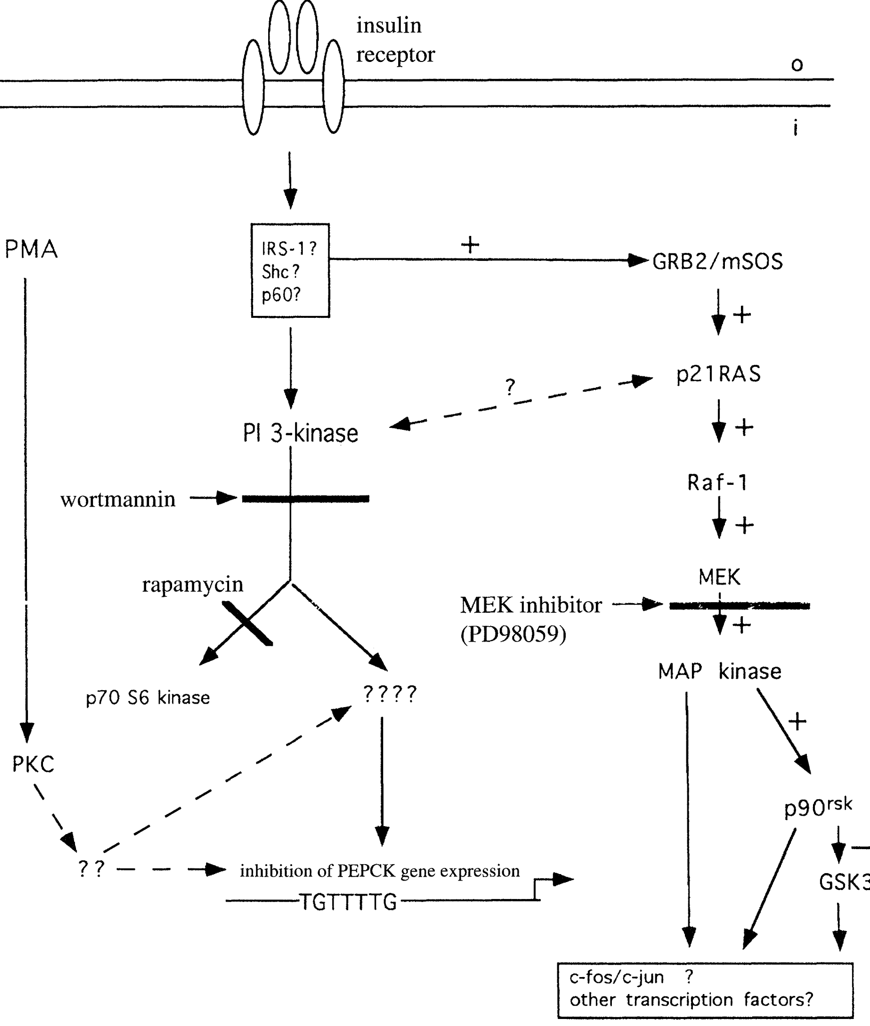

Figure 3. New connections in insulin-regulated gene expression. Abbreviations are as follows. PMA phorbol 12-myristate 13-acetate; IRS-1 insulin receptor substrate-1; PI 3-kinase phosphatidylinositol 3-kinase; GSK3 glycogen synthase kinase-3; MAP kinase Mitogen Activated Protein kinase; MEK MAP kinase/ERK kinase; p70 S6 kinase p70/p85 ribosomal protein S6 kinase; $p90^{rsk}$ p90 ribosomal S6 protein kinase.

tional activation of c-fos by this hormone (Lazar *et al.* 1995). In contrast this compound had no effect on the regulation of PEPCK gene expression by insulin in the HL1C cells (see figure 4), despite completely blocking the activation of MAP kinase in these cells (B. Gabbay *et al.* 1995). In addition, inhibitors of $p21^{ras}$ farnesylation or expression of a dominant negative $p21^{ras}$ ($N17^{Ras}$), both of which blocked activation of MAP kinase by insulin, has no effect on insulin action on PEPCK gene transcription in the HL1C cells (B. Gabbay *et al.* 1995). The MAP kinase pathway is also activated by phorbol esters in a number of cell types (Northwood & Davis 1990; Chung *et al.* 1991; Tobe *et al.* 1991; de Vries-Smits *et al.* 1992). This effect of phorbol esters may be mediated by an activation of Raf-1, possibly through direct phosphorylation by PKC (Chao *et al.* 1994; Marquardt *et al.* 1994). However, like insulin, phorbol ester-regulated PEPCK gene expression is not affected by inhibition of MEK (see figure 4). Thus the signalling molecules required for regulation of PEPCK gene expression by insulin and phorbol esters, are distinct from those of the p42/p44 MAP kinase pathway. Of course it is not yet known whether other MAP kinase isoforms (see, for example, p38/p46/p54 in Davis 1994) can be activated in liver cells by insulin, and whether PD98059 would block such an activation. Thus a role for a distinct MAP kinase-related pathway in insulin-regulated PEPCK gene expression cannot yet be excluded.

3. INSULIN SIGNALLING AND PHOSPHATIDYL INOSITOL 3-KINASE

As described above, insulin can activate the $p21^{ras}$/MAP kinase pathway through the binding of GRB-2 to tyrosine phosphorylated IRS-1 (Myers *et al.* 1994*b*; White & Kahn 1994; Denton & Tavare 1995). However, IRS-1 can be phosphorylated at up to twenty distinct tyrosine residues, therefore it can potentially act as a docking protein for a number of SH2-containing proteins. In fact, several other IRS-1 associated proteins have already been identified, including phosphatidylinositol (PI) 3-kinase (Sun *et al.*

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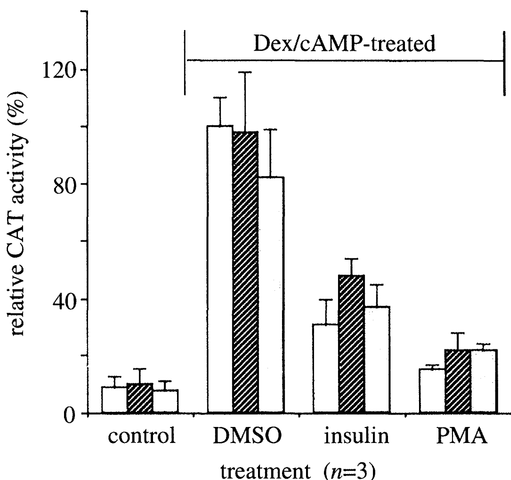
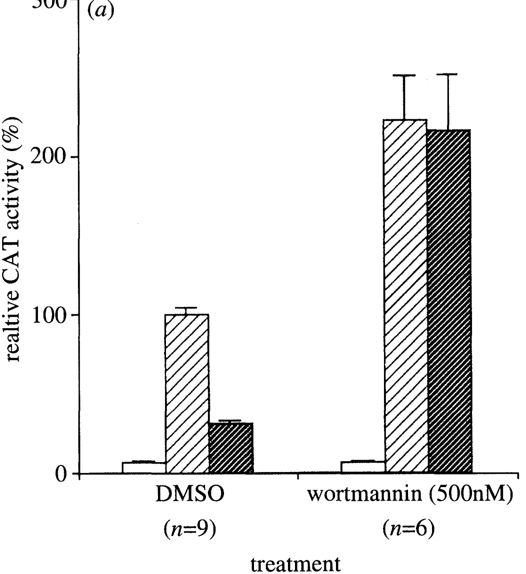


Figure 4. Inhibition of MEK does not affect the action of insulin or phorbol esters on PEPCK-CAT gene expression. HL1C cells were incubated in serum-free medium containing 500 nM dexamethasone and 0.1 mM 8CPT-cAMP (Dex/cAMP-treated) along with 10 nM insulin (insulin), 1 μM PMA (PMA), or 0.05% DMSO (DMSO), in the presence of 0, 1, or 10 μM MEK inhibitor PD98059. As a control HL1C cells were incubated with 0.05% DMSO in the presence of 0, 1, or 10 μM MEK inhibitor PD98059 (control). After 3 h at 37 °C the cells were harvested and assayed for CAT activity as described previously (Sutherland *et al.* 1995). Results are expressed as % CAT activity relative to Dex/cAMP stimulated activity in the absence of inhibitor. Results represent the mean ± standard error of the number of experiments shown in parenthesis. Open area: no inhibitor; hatched area: 1 μM inhibitor; shaded area: 10 μM inhibitor.

1991; Backer *et al.* 1992), nck (Lee *et al.* 1993) and Syp (Kuhne *et al.* 1993; Sun *et al.* 1993). Thus the formation of the IRS-1 : GRB-2 complex may initiate only one of several insulin-stimulated signal transduction pathways.

PI 3-kinase is a heterodimer, containing a 110 kDa (p110) catalytic subunit and a 85 kDa (p85) regulatory subunit. The p85 subunit interacts with tyrosine phosphorylated IRS-1 via two SH2 domains and this causes an activation of the associated p110 subunit (Backer *et al.* 1992). Activation of PI 3-kinase leads to the accumulation of phosphoinositols *in vivo*, mainly phosphatidylinositol 3,4,5 trisphosphate (Auger *et al.* 1989). This phospholipid has been postulated to act as a 'second messenger' molecule (Auger *et al.* 1989), although its *in vivo* targets are still unknown (Nakanishi *et al.* 1993; Toker *et al.* 1994). PI 3-kinase activity is blocked by the antibiotic, wortmannin, which exhibits an IC-50 of 1–10 nM *in vitro* (Arcaro & Wymann 1993). The activation of PI 3-kinase must be important in insulin action because wortmannin blocks the antilipolytic action of insulin in adipocytes (Rahn *et al.* 1994), the inhibition of glycogen synthase kinase-3 (GSK3) by insulin (Cross *et al.* 1994; Welsh *et al.* 1994), the inhibition of apoptosis by insulin (Yao & Cooper 1995), and insulin-stimulated membrane ruffling (Kotani *et al.* 1994), glucose uptake (Clarke *et al.* 1994; Gould *et al.* 1994; Shimizu & Shimazu 1994; Yeh *et al.* 1995) and glycogen synthesis (Shepherd *et al.* 1995; Yamamoto-Honda *et al.* 1995).

Wortmannin completely blocks the regulation of PEPCK-CAT gene expression by insulin (see figure 5*a*), as well as the regulation of endogenous PEPCK



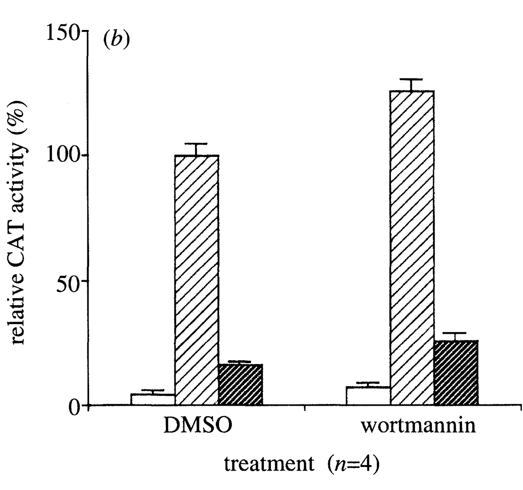


Figure 5. Insulin signalling, but not phorbol ester signalling, to the PEPCK promoter is wortmannin-sensitive. HL1C cells were incubated in serum-free medium in the absence of hormone (control), in 500 nM dexamethasone and 0.1 mM 8CPT-cAMP (Dex/cAMP), in 500 nM dexamethasone, 0.1 mM 8CPT-cAMP and (*a*) 10 nM insulin (Dex/cAMP/insulin), or in 500 nM dexamethasone, 0.1 mM 8CPT-cAMP and (*b*) 1 μM PMA (Dex/cAMP/PMA). Wortmannin (500 nM) or DMSO carrier was included in each hormone treatment and the cells were harvested after 3 h incubation (as described in Sutherland *et al.* 1995). The results are expressed as the % CAT activity relative to that obtained with Dex/cAMP alone. Results represent the mean ± standard error of the number of experiments shown in parenthesis. Open area: control; wide-hatched area: Dex/cAMP; close hatched area: Dex/cAMP/insulin.

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gene transcription by insulin in the HL1C cells (Sutherland *et al.* 1995). Recently, a mechanistically distinct inhibitor of PI 3-kinase, LY294002 (Vlahos *et al.* 1994), was also shown to block insulin signalling to the PEPCK promoter (B. Gabbay *et al.* 1995). These data demonstrate that PI 3-kinase is required for the regulation of PEPCK gene expression by insulin (see figure 3). In contrast, wortmannin had no effect on PMA-regulated PEPCK-CAT gene expression (see figure 5*b*), again demonstrating either the existence of distinct signalling pathways for insulin and PMA, or activation, by a PMA-dependent process, of a molecule which functions downstream of PI 3-kinase (see figure 3).

Wortmannin blocks the activation of p70/p85 ribosomal S6 protein kinase ($p70^{s6k}$) by insulin (Cheatham *et al.* 1994; Yamamoto-Honda *et al.* 1995). $p70^{s6k}$ is the protein kinase responsible for insulin- and growth factor-stimulated phosphorylation of ribosomal protein S6 *in vivo* (Chung *et al.* 1992; Price *et al.* 1992). The macrolide rapamycin is an inhibitor of insulin-stimulated $p70^{s6k}$ activity (Chung *et al.* 1992; Sutherland *et al.* 1995) and ribosomal protein S6 phosphorylation (Chung *et al.* 1992). The mechanism of $p70^{s6k}$ activation and the direct target of rapamycin remain unclear (for a review see Ferrari & Thomas 1994), however rapamycin has no effect on the stimulation of PI 3-kinase by insulin. The regulation of PEPCK gene expression by either insulin or PMA was not affected by rapamycin (Sutherland *et al.* 1995). Thus, although the activation of $p70^{s6k}$, and the regulation of PEPCK gene expression by insulin both require PI 3-kinase, they lie on distinct downstream signalling pathways (see figure 3).

4. BRIDGING THE GAP BETWEEN PI 3-KINASE AND THE PEPCK PROMOTER

As mentioned above, the phosphorylated lipids produced by the action of PI 3-kinase may act as second messengers. Indeed, *in vitro*, phosphatidylinositol-3,4,5 trisphosphate, may directly activate the phorbol ester-insensitive isoform of protein kinase C, termed PKCζ (Nakanishi *et al.* 1993). Toker *et al.* recently reported that other phorbol ester-insensitive PKC isoforms are activated by PI-3,4,5 trisphosphate to a greater extent than PKCζ (Toker *et al.* 1994). Thus a role for one or more of these phorbol ester-insensitive forms of PKC in insulin signalling remains a possibility. PI 3-kinase appears to be tightly associated with, or contains, a protein kinase activity (Backer *et al.* 1992; Carpenter *et al.* 1993; Dhand *et al.* 1994), thus novel protein substrates may yet be found for this enzyme, one or more of which may mediate insulin's action on PEPCK gene transcription.

The small G protein, rac 1 (a member of the rho family of G proteins), may lie downstream of PI 3-kinase in a signal transduction cascade (Hawkins *et al.* 1995). Although rac-1 might mediate regulation of cytoskeletal rearrangements such as membrane ruffling and exocytosis (Ridley *et al.* 1992; Kishi *et al.* 1993; Takaishi *et al.* 1993; Tominaga *et al.* 1993; Price *et al.* 1995), it is possible that other functions, including regulation of gene expression, may yet be identified for this molecule. Indeed, rac-1 has recently been found to stimulate the autophosphorylation of a serine/threonine protein kinase, termed PAK65 (Martin *et al.* 1995). The kinase domain of PAK65 is homologous to that of STE20, a yeast protein kinase involved in the activation of the yeast MAP kinase homologues FUS3/KSS1 (Errede *et al.* 1993; Errede & Levin 1993). However, any connection between the activation of PAK65 and the transcription of a specific gene remains to be identified.

Other growth factors also stimulate PI 3-kinase activity, and the study of transcription factors regulated by these agents could aid in the elucidation of insulin signalling pathways. For example epidermal growth factor (EGF) mimics the action of insulin on the inhibition of cAMP-stimulated PEPCK expression in primary hepatocyte cultures (Molero *et al.* 1992; Fillat *et al.* 1993). It is not known whether wortmannin blocks this action of EGF. The inhibition of cAMP-stimulated PEPCK gene expression by EGF is partially attenuated by prolonged exposure to phorbol esters, thus PKC may play a role in this action of EGF (Fillat *et al.* 1993).

Due to the apparent complexity of the signal transduction pathways partly identified to date, it may be that the simplest method for mapping a linear signalling pathway from the insulin receptor to the PEPCK promoter will be to begin with the identification of the transcription factor(s) that bind specifically to IRS(s) within the PEPCK promoter, followed by the identification of molecules that interact with or modify these factors. Unfortunately this has not been a trivial task because, although several proteins bind to the PEPCK IRS *in vitro*, none have demonstrated the necessary correlation between binding and function (with respect to insulin action) (O'Brien *et al.* 1994*a*, 1995). Thus, despite the identification of several new connections in insulin-regulated gene expression, it would seem that researchers working at either end of the signal transduction pathway between PI 3-kinase and the PEPCK IRS's still have much left to accomplish.

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Discussion

J. Saklatvala (*Cytokine Laboratory, The Babraham Institute, Cambridge, U.K.*). Might the reported insensitivity of Professor Granner's effects to inhibition by the phosphoinositide 3-kinase inhibitors be because a small proportion of the total activity is all that is needed, and that this much survives inhibition?

D. K. Granner. We tested the inhibitors against the kinase and they appeared to be effective, but we could not rule out a very small residual activity.

P. Cohen (*University of Dundee, U.K.*). Was not the concentration of the Parke-Davis inhibitor that Professor Granner used a little low for maximal effects? *In vitro*, its K_i is about 2 μM and he used 10 μM?

D. K. Granner. We based the concentrations we used on effective levels reported in work by others. If we put in more, it killed the cells. We have also checked that this concentration does indeed inhibit the MAP kinase activity in our cells.

P. Cohen. There are also difficulties in interpreting the actions of this inhibitor. It is reported in the literature that it inhibits MAP kinase kinase, but it does not. In fact, it binds to the inactive form of MAP kinase kinase and thereby prevents its activation by Raf. We have found that it always blocks the activity of MAP kinase kinase by 80–90%. Whether that prevents the activation of MAP kinase depends on the strength of the signal. For example, insulin is a weak MAP kinase activator so the inhibitor blocks its effect completely, whereas the much more potent activation by phorbol ester is often only partly inhibited.

M. F. White. My question is about insulin-stimulated protein synthesis. In our hands this involves IRS-I and is inhibited by wortmannin, and this inhibition can be rescued by TPA (by an unknown mechanism). Could some of the effects reported by Professor Granner be due to wortmannin inhibition of general protein synthesis?

D. K. Granner. The control of PEPCK transcription by insulin is not blocked by cycloheximide, puromycin or anisomycin, and the stimulatory effects of insulin on run-on transcription assays can be picked up within only a minute or two, so it is clear that the short-term control of transcription by insulin does not require protein synthesis.