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Signal transduction convergence: Phorbol esters and insulin inhibit phospho*enol*pyruvate carboxykinase gene transcription through the same 10-base-pair sequence

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ABSTRACT **Phospho*enol*pyruvate carboxykinase (PEPCK) governs the rate-limiting step in gluconeogenesis. Glucocorticoids and cAMP increase PEPCK gene transcription and gluconeogenesis, whereas insulin and phorbol esters have the opposite effect. Insulin and phorbol esters are dominant, since they prevent cAMP and glucocorticoid-stimulated transcription. Basal promoter elements and hormone response elements for cAMP, glucocorticoids, and insulin have been defined in previous studies. By using stable transfectants containing a variety of different PEPCK–chloramphenicol acetyltransferase fusion gene constructs, a phorbol ester response sequence, located between positions −437 and −402 relative to the transcription start site, was identified. This region coincides with the insulin response sequence that has recently been defined in the PEPCK promoter. Using a vector containing various wild-type and mutated sequences of this region ligated to the heterologous thymidine kinase promoter, we delineated the boundaries of both elements to the 10 base pairs between positions −416 through −407. Thus, although it has been previously shown that insulin and phorbol esters repress PEPCK gene transcription through distinct pathways, the final target of insulin and phorbol ester action is the same DNA element.**

One of the intriguing things about the many genes regulated by insulin or phorbol esters is that the gene products affect a diversity of biological phenomena and include metabolic enzymes, secretory proteins/hormones, integral membrane proteins, and transcription factors (1, 2). In some cases phorbol esters and insulin affect the expression of the same gene. Both of these effectors stimulate c-*fos* and gene *33* transcription (3–6), whereas both of them inhibit transcription of phospho*enol*pyruvate carboxykinase (PEPCK) (7–9). Genes whose transcription is regulated in opposing directions by insulin and phorbol esters have yet to be identified.

Phorbol esters and insulin decrease hepatic gluconeogenesis by inhibiting basal and glucocorticoid- or cAMP-stimulated transcription of the PEPCK gene (7–9). Although the initial actions of phorbol esters and insulin on PEPCK gene transcription are clearly separable (10, 11), the fact that both result in a very rapid reversible inhibition that is independent of on-going protein synthesis (7, 8) suggests a common final pathway. The basal promoter elements, the cAMP response element, the glucocorticoid response unit (GRU), and the insulin response sequence (IRS) have been mapped in an effort to understand the complex regulation of the PEPCK gene (12–15). Several cis-acting elements, including the serum response element and the binding sites for transcription factors Jun/AP-1, AP-2, AP-3, and NFκB, mediate transcriptional activation by phorbol esters (16–22), but no inhibitory cis-acting element has been identified. We describe such an element here and in so doing report an example of signal transduction convergence: the inhibitory effects of phorbol esters and insulin on the PEPCK gene, which start with the generation of unique signals, are mediated through a common 10-base-pair (bp) sequence.

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MATERIALS AND METHODS

Plasmid Construction. The construction of a series of reporter constructs containing 5′ deletion mutations of the PEPCK promoter ligated to the chloramphenicol acetyltransferase (CAT) gene has been described (23). Plasmid TKC-VI (provided by T. Sudhof, Southwestern Medical School, Dallas) contains the herpes simplex virus thymidine kinase (TK) promoter ligated to the CAT gene (24). The TK promoter sequence extends from positions −480 to +51 and contains a *Bam*HI linker between positions −48 and −32 (15). Various complementary oligonucleotides (see Fig. 1) were synthesized with *Bam*HI compatible ends using an Applied Biosystems 380A DNA synthesizer and cloned in either orientation into *Bam*HI-cleaved TKC-VI. All plasmid DNA constructs were sequenced and centrifuged twice through cesium chloride gradients.

Cell Culture and Transfection. H4IIE rat hepatoma cells were grown in Dulbecco's modified Eagle's medium containing 2.5% (vol/vol) newborn calf serum and 2.5% (vol/vol) fetal calf serum. The establishment of a series of stably transfected cell lines and transient transfection protocols has been described (15, 25).

CAT Assay. Transiently or stably transfected cells were harvested by trypsin digestion and sonicated in 250 μl of 250 mM Tris·HCl (pH 7.8). Samples were then heated to 60°C for 10 min and centrifuged briefly to remove cellular debris. CAT activity in the supernatant was assayed by a modification (14) of the method of Nordeen *et al.* (26). Background activity from a lysate-free blank was subtracted from all assays. CAT activity was corrected for the protein concentration in the lysate, as measured by the Pierce BCA assay.

RESULTS

Insulin Inhibits PEPCK Gene Transcription Through a 10-bp Sequence. We recently used the TKC-VI vector, which contains the herpes simplex virus TK promoter ligated to the CAT reporter gene, to identify an IRS in the PEPCK promoter (15). A 15-bp double-stranded oligomer (PC425), which has the wild-type PEPCK sequence between positions −416

Abbreviations: PEPCK, phospho*enol*pyruvate carboxykinase; PMA, phorbol 12-myristate 13-acetate; IRS, insulin response sequence; PRS, phorbol ester response sequence; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; GRU, glucocorticoid response unit.

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and −402 ligated into the *Bam*HI site of TKC-VI, conferred an insulin-dependent orientation-independent inhibition of CAT expression in transient transfection assays (Table 1 and ref. 15). A 5-bp mutation within this region, designated PC4M5, was constructed by altering the sequence between positions −411 and −407. This mutation abolished the insulin effect (Table 1 and ref. 15). Another mutation, designated PCM2M, was then constructed by altering the 5-bp sequences between positions −416 and −412 and between positions −406 and −402 on either side of the wild-type sequence between positions −411 and −407. This mutation also failed to give an insulin-dependent inhibition of CAT expression in either orientation (Table 1 and ref. 15). Several specific protein–DNA complexes were detected when the 15-bp wild-type sequence between positions −416 and −402 (PC425) was used as the labeled probe in a gel-retardation assay (15). Sequences containing mutations that resulted in a loss of function in response to insulin (PC4M5 and PCM2M) failed to compete with the wild-type sequence for binding to the factor(s) (15).

Continuing this analysis, separate 5-bp mutations of the sequences between positions −416 and −412 and between positions −406 and −402 were constructed; these were designated PCM25 and PC42M, respectively. These constructs were inserted into the TKC-VI vector and the transient expression of CAT activity was analyzed in H4IIE cells. Though plasmids containing either orientation of the PC42M construct still responded to insulin, the PCM25 mutation abolished the insulin effect (Table 1). A smaller construct (PC42), consisting only of the 10-bp wild-type PEPCK sequence between positions −416 and −407, showed insulin-dependent inhibition of CAT expression in the transient transfection assay. A mutation in PC42 equivalent to that in PC4M5, designated PC4M, abolished the inhibitory effect of insulin, as did the identical mutation in the 15-bp oligomer.

Ligation of multiple (four) copies of the PC42 construct into the TKC-VI vector augmented the effect of insulin on the transient expression of CAT activity by this construct (Fig. 1*A*). Insulin at 10 nM almost completely inhibited basal CAT expression by this construct. This result was confirmed by primer-extension analysis, using calmodulin as a hormonally unresponsive internal control, in which insulin (10 nM) caused a 69% reduction in correctly initiated CAT mRNA (data not shown).

Table 1. Use of the herpes simplex virus TK promoter to delineate a PEPCK gene IRS

Designation	Construct	Effect of insulin on basal CAT expression, % change: Correct orientation	Effect of insulin on basal CAT expression, % change: Inverted orientation
	−411 −407		
PC425	TGGTGTTTTGACAAC	−58	−44
PC4M5	TGGTGGGGGTACAAC	+1	0
PCM2M	GTCCCTTTTGTTAGC	+3	+2
PC42M	TGGTGTTTTGTTAGC	−41	−40
PCM25	GTCCCTTTTGACAAC	+24	+3
PC42	TGGTGTTTTG	−53	−32
PC4M	TGGTGGGGGT	+19	+21

Various mutants of the PEPCK gene promoter sequence between positions −416 and −402 were synthesized with *Bam*HI (GATC) ends and cloned, in either orientation, into the TKC-VI vector. The effect of insulin on CAT expression was analyzed by transient transfection of rat hepatoma H4IIE cells. Transfected cells were incubated in serum-free medium in the presence or absence of insulin and harvested after 18 hr; CAT activity was assayed. Results are the ratio of CAT activity in insulin-treated versus control cells (expressed as percent change) and represent the mean of 6–14 transfections for each construct. Maximum error (±SEM) was 6%. Underlined sequences are the mutant sequences. The vector without an insert showed a slight stimulation in response to insulin (+19 ± 6%; n = 12).

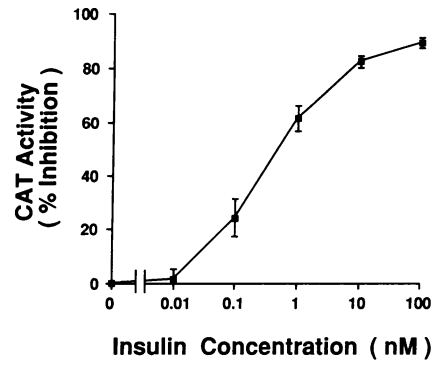

FIG. 1. Multiple copies of the PEPCK IRS augment the insulin response. Four copies of the PC42 construct (PEPCK sequence between positions −416 and −407) were ligated into the *Bam*HI site of TKC-VI. The effect of various concentrations of insulin on CAT expression was analyzed by transient transfection of H4IIE cells. Transfected cells were harvested after an 18-hr incubation in serum-free medium in the presence or absence of various concentrations of insulin. CAT activity was assayed and results are expressed as the percent inhibition of basal CAT activity by insulin and represent the mean ± SEM of eight transfections.

The PEPCK Promoter Contains a Cis-Acting Phorbol Ester Response Sequence (PRS). Phorbol esters and insulin have similar effects on PEPCK gene transcription. The action of both agents is inhibitory, exerted rapidly, readily reversible, independent of on-going protein synthesis, and dominant over the stimulatory actions of dexamethasone or cAMP (7, 8). Given these similarities, and having shown that the 10-bp sequence between positions −416 and −407 is a functional IRS, we sought to determine whether phorbol esters functioned through this same element. The initial test system utilized a line of H4IIE cells (HL1C) that is stably transfected with a PEPCK–CAT construct containing the PEPCK promoter sequence between positions −2100 and +69 (25). The regulation of the transfected gene in these cells by cAMP, dexamethasone, and insulin mimicked that of the endogenous gene (8, 25). Like insulin, phorbol 12-myristate 13-acetate (PMA) prevented the increase of CAT activity caused by cAMP, dexamethasone, or a combination of both agents (Fig. 2*A*). Thus, the PEPCK promoter contains a cis-acting PMA-responsive silencing element and, as with the endogenous gene, the inhibitory action of PMA was dominant. The stimulation of CAT expression by dexamethasone and cAMP in the HL1C stable transfectant was inhibited in a concentration-dependent manner in response to PMA; the half-maximal effective concentration was about 10 nM and maximal inhibition was obtained with 1 μM PMA (Fig. 2*B*), concentrations that provide similar degrees of inhibition of the endogenous gene (7).

Localization of a PRS in the PEPCK Promoter. A series of H4IIE cell lines stably transfected with various PEPCK–CAT fusion genes was analyzed in an effort to delineate a PMA response sequence (PRS) (Table 2). PMA had the same effect on expression of CAT activity in the HL1, HL9, FBG32, and HL10 stable transfectants as it has on the endogenous PEPCK gene; it prevented the stimulation by cAMP and dexamethasone (Table 2 and ref. 7). However, in the HL12 and HL45 stable transfectants the effect of PMA was clearly reduced. These results suggest that the PEPCK promoter contained at least a portion of a PRS between positions −437 and −402. Since this region also contains the

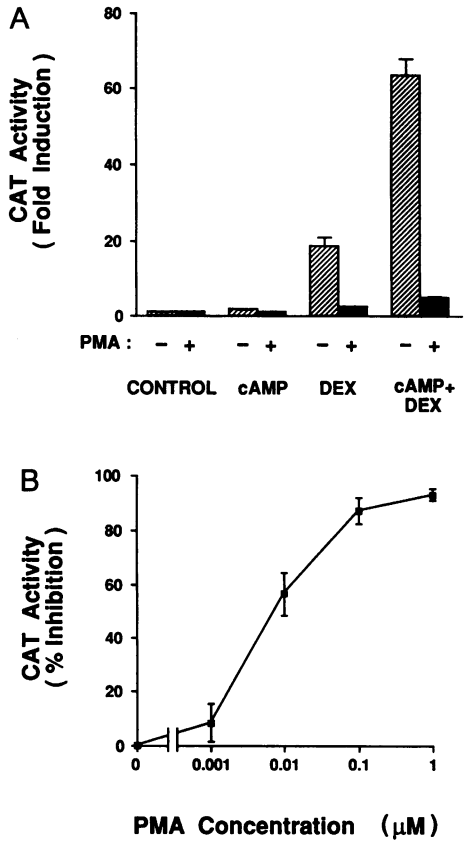

FIG. 2. Multihormonal regulation of PEPCK–CAT fusion genes. H4IIE cells were stably transfected with the plasmid pPL1-CAT, which contains the region of the PEPCK promoter from positions −2100 to +69. Cells were incubated for 18 hr in serum-free medium with dexamethasone (DEX; 500 nM) and 8-(4-chlorophenylthio)-cAMP (cAMP; 0.1 mM) in the absence (−) or presence (+) of 1 μM PMA (*A*) or various concentrations of PMA (*B*). (*A*) Results are expressed relative to CAT activity in untreated cells. (*B*) Data represents the percent inhibition by PMA of CAT activity induced by 500 nM dexamethasone and 0.1 mM cAMP. Results represent the mean ± SEM of five experiments.

IRS, we postulated that PMA may function through the same 10-bp sequence (positions −416 to −407) as does insulin. In subsequent experiments no response to PMA was noted when the TKC-VI vector or the vector containing the mutant PC4M construct was transiently transfected into H4IIE cells. However, the vector containing the PC42 construct did show a PMA-responsive inhibition of CAT expression (Fig. 3).

DISCUSSION

The hormone response element paradigm implies that discrete segments of DNA confer the response of a hormone in a heterologous system and that this DNA sequence interacts specifically with one or more proteins that are involved in regulating transcription. We have localized a functional PMA-response sequence (PRS) in the PEPCK promoter to the region between positions −416 and −407. This PRS functions as a silencing element when attached to a heterologous promoter, and its sequence coincides with that of an IRS. Although the serum response element in the c-*fos* gene promoter mediates transcriptional activation by insulin and phorbol esters (16, 27), the element described herein bears no homology with the serum response element or with any other previously identified stimulatory phorbol ester response elements (16–22).

The IRS/PRS element may be unique to the PEPCK promoter, as is the GRU through which glucocorticoids

Table 2. Multihormone regulation of PEPCK–CAT fusion genes

PEPCK–CAT construct 5′ end, position	Clone	% inhibition of CAT activity: Induction by DEX and cAMP, fold increase	% inhibition of CAT activity: Inhibition by PMA, %
−2100	HL1C	63.5 ± 4.4	94 ± 1
−600	HL9L	13.8 ± 1.0	74 ± 2
	HL9J	3.2 ± 0.2	89 ± 4
−468	FBG32B	7.2 ± 1.3	71 ± 3
−437	HL10J	65.9 ± 5.5	90 ± 1
−402	HL12E, HL12D, HL12K, HL12MIX	3.8 ± 0.7	44 ± 12
−271	HL45I	5.6 ± 1.2	20 ± 7

H4IIE cells were stably transfected with plasmids containing various PEPCK 5′ deletion mutations. Cells were incubated for 18 hr in serum-free medium containing dexamethasone (DEX; 500 nM) and 8-(4-chlorophenylthio)-cAMP (cAMP; 0.1 mM) in the absence or presence of 1 μM PMA and CAT activity was assayed. Data are the mean ± SEM of five experiments. The regulation of these stably transfected genes by dexamethasone and cAMP is qualitatively similar to that of the endogenous gene but quantitatively the cAMP response is reduced and the dexamethasone response is variable—compare HL9L and HL9J.

stimulate PEPCK gene transcription (ref. 14 and see below). However, sequences similar to the IRS/PRS are present in a number of other insulin-regulated genes (Table 3). Gene *33* transcription is regulated by both insulin and PMA (5, 6); however, whether the other genes shown in Table 3 are also regulated by phorbol esters is not known. Though it is interesting that sequences similar to the IRS/PRS are present in both genes whose transcription is inhibited and stimulated by insulin, in most cases it remains to be determined whether these similarities are functionally significant. In the δ1-crystallin gene this similarity is probably not functionally significant (29); however, the sequences shown from the amylase, insulin-like growth factor binding protein 1, and aspartate aminotransferase genes are within functionally

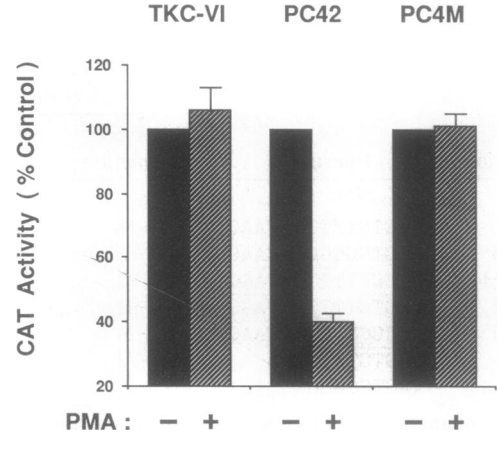

FIG. 3. Sequence between positions −416 and −407 responds to PMA. The effect of PMA on CAT expression directed from the native TKC-VI vector or the vector containing the PC42 or PC4M constructs was analyzed by transient transfection. Transfected cells were harvested after a 9-hr incubation in serum-free medium in the presence (+) or absence (−) of PMA (1 μM). CAT activity was quantitated and results are expressed as a percentage of the CAT activity in insulin-treated versus control cells and represent the mean ± SEM of seven transfections for each construct. The PC42 construct responded equally well to PMA when inserted into the TKC-VI vector in either orientation (data not shown).

Table 3. The 10-bp sequence between positions −416 and −407 is present in other insulin-regulated genes

Gene	5′ end, position	Sequence	No. match/ no. total	Effect of insulin	Ref(s).
PEPCK	−416	TGGTGTTTTG	10/10	I	8, 9, 28
Aspartate aminotransferase	−1374	TGGTGTTTTG	10/10	I	PC
δ1-Crystallin	−1692	TGGTGTTCTG	9/10	S	29, 30
Gene 33	−954	TGGCGTTTTG	9/10	S	6, 31
Adipsin	−75	TGGTTTTCTG	8/10	I	32, 33
Glucokinase	−83	TGGTTCTTTG	8/10	S	34
PF-2-K/F-2,6-BP	−166	TGTGGTTTTG	8/10	S	35, 36
Malic enzyme	−692	TATTGTTTTG	8/10	S	37, 38
α-Amylase	−165	GTTTATTTTG	6/10	S	39, 40
IGFBP-1	−284	TGTCTTTTTG	7/10	I	41, †

All the sequences shown are from the coding strand of the respective gene promoter except that for aspartate aminotransferase (M. Aggerbeck and R. Barouki, Paris, personal communication). 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase is abbreviated PF-2-K/F-2,6-BP. Locations are expressed relative to the transcription start site. The effect of insulin on the expression of these genes is shown as inhibition (I) or stimulation (S). In every case, apart from amylase and insulin-like growth factor binding protein 1 (IGFBP-1), the effect of insulin has been shown, using a "run-on" assay, to be at the transcriptional level. Underlined bases represent differences from the PEPCK sequence.

defined insulin responsive regions (40, †). The relatively poor sequence homology may indicate some variability in the precise sequence recognized by the IRS/PRS binding protein(s) similar to the situation with the transcription factor C/EBP (43).

Evidence from two independent assays suggests that one or more proteins in liver and H4IIE hepatoma cell nuclear extracts recognize the PEPCK gene PRS/IRS. A DNase I footprint spans this region (14), and a complex pattern of binding of nuclear proteins is detected by gel-mobility-shift analysis (15). Though phorbol esters induce binding of the transcription factor NF-κB (22), no change in the IRS/PRS gel-retardation pattern was detected in comparisons of nuclear extracts from control and phorbol ester-treated H4IIE cells (unpublished observations).

Tumor-promoting phorbol esters, thought to act by activating protein kinase C (10), have some insulinomimetic effects, including the stimulation of hexose transport (44, 45), lipogenesis (46), and the activation of pyruvate dehydrogense (45) and gene *33* transcription (5). Insulin is thought to initiate its actions by activating its receptor tyrosine kinase, but whether it also stimulates protein kinase C is controversial (47). Preincubation of cells with phorbol esters down-regulates some, but not all, protein kinase C isozymes (48). In H4IIE cells such a treatment attenuates PMA-inhibited PEPCK gene transcription but does not affect the inhibitory action insulin has on this gene (49). Thus, the inhibitory effect of insulin is mediated either by a signal transduction pathway independent of protein kinase C or through a protein kinase C isozyme that is not down-regulated by PMA pretreatment. In either case, the pathways used by insulin and phorbol esters to inhibit PEPCK gene transcription are distinct, though the final target of phorbol ester and insulin action is a single DNA element in the PEPCK gene promoter.

How might this IRS/PRS function in the context of the PEPCK promoter to inhibit glucocorticoid-induced transcription? A complex GRU mediates the stimulatory action of glucocorticoids on the PEPCK gene (14). This GRU consists of a tandem array (5′ to 3′) of two accessory factor binding sites (AF1 from positions −455 to −431 and AF2 from positions −420 to −403) and two glucocorticoid receptor binding sites (GR1 and GR2 from positions −395 to −349). AF1 and AF2 are both essential components of the GRU. Thus, since the IRS/PRS coincides with AF2, it is ideally positioned to inhibit AF2 function and this could explain how insulin and phorbol esters mediate their dominant negative effect on glucocorticoid-stimulated PEPCK gene transcription. Whether the same protein(s) mediate the response to all these effectors in the PEPCK gene is unknown, but mutations that disrupt the response to phorbol esters or insulin concommitantly disable the accessory factor function of AF2 in response to glucocorticoids (J. Mitchell and D.K.G., unpublished observations).

Hormone response elements can be simple (50), but recent evidence suggests that various combinations of cis-acting elements, often involving protein–protein interactions between apparently unrelated transcription factors, form complex regulatory domains (42, 51–53). The PEPCK gene promoter represents an example of this emerging paradigm in which multiple effectors converge on a relatively short segment of DNA. The dissection of this domain should provide a molecular genetic explanation for the complex hormonal regulation of gluconeogenesis.

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