BIOCHEMISTRY

Insulin and Phorbol Ester Regulation of Gene 33 Expression in CHO Cells

NANCY ROMERO, MS*; BRAULIO D. JIMÉNEZ, PhD†; CARMEN L. CADILLA, PhD†

ABSTRACT. Rat gene 33 (g33) mRNA has a widespread tissue distribution. Insulin and various agents such as glucocorticoids, phorbol esters and plant lectins regulate G33 expression in rat hepatoma cells. The regulation of g33 by insulin and a phorbol ester was examined in two Chinese Hamster ovary (CHO) cell lines, CHO-T cells (which overexpress human insulin receptors (hIR)) and wild type CHOwt cells. These cell lines were used to determine how expression of the hIR influences the capacity of g33 to respond to insulin and phorbol myristate acetate (PMA). Treatment of CHOwt and CHO-T cells with insulin increased mRNA^{g33} levels three to four-fold, with a maximum effect reached after three hours of treatment. PMA treatment of CHOwt and CHO-T cells caused a similar elevation of mRNA^{g33} levels after three hours. Insulin had no effect on mRNA^{g33} stability in both CHO cell lines. Additionally, the effects of insulin and PMA on mRNA^{g33} levels were additive only in CHO-T cells. Insulin or PMA-pretreated CHO-T cells were able to respond to both agents, but elevation of mRNA^{g33} levels was submaximal. In contrast, when insulin and/or PMA-pretreated CHOwt cells were exposed to insulin or PMA, g33 was able to respond maximally. These results suggest that insulin and phorbol esters act through different signaling mechanisms in CHOwt cells. Additionally, insulin's ability to stimulate g33 expression in CHOwt cells suggests that this insulin effect may be independent of the insulin receptor. There are differences in the regulation pattern of g33 by insulin and PMA in rat hepatoma and among the two CHO cell lines used in this study. *Key Words: Insulin, Insulin receptor, Phorbol myristate acetate, Gene 33, CHO, Hormonal regulation*

The expression of many eukaryotic genes is influenced by the activity of intracellular signal transduction pathways. Insulin, the vital pancreatic polypeptide hormone, may have an important role as a growth factor in certain tissues during embryogenesis and later, during hepatic regeneration. Unfortunately, the molecular basis of many of insulin's actions remains largely unsolved, primarily because the key element(s) of signal transduction are not completely understood.

From the University of Puerto Rico, School of Medicine, Departments of Microbiology and Medical Zoology* and Biochemistry†, San Juan, Puerto Rico.

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Address for correspondence: Dr. Carmen L. Cadilla, University of Puerto Rico, School of Medicine, Department of Biochemistry, PO Box 365067, San Juan, PR 00936-5067 USA. Tel.: 1-787-754-4366; FAX: 1-787-274-8724; Email:c_cadilla@rcmaca.upr.clu.edu

The insulin receptor is unlike other receptor tyrosine kinases since it mostly utilizes docking proteins such as IRS1/2 (1,2) instead of binding directly to SH2 domain-containing signaling proteins. IRS1 interacts with several SH2 domain-containing proteins, including Grb2, the p85 α/ß subunit isoforms of phosphatidylinositol-3'-kinase, the phosphatase SHTP2 or Syp, and the adaptors Nck and Crk (1). IRS1/2 plays a central role in linking the activation of the insulin receptor activated tyrosine kinase to the myriad of effects caused by insulin.

The inositol phosphate/protein kinase C (PKC) pathway has been postulated to play a role in insulin action partly due to the fact that this signal transduction pathway is a major component of the response to ligands such as insulin, which bind to receptors with intrinsic tyrosine kinase activity, and that phorbol esters or synthetic diacylglycerols can mimic many cellular actions of insulin. Evidence from Blackshear's laboratory suggests that the common PKC species are not involved in a major way (3).

One of the best-studied insulin-regulated genes is the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C) (reviewed in ref. 4), a gene which provides an excellent model for metabolic control of gene expression. Insulin has a dominant and negative effect on PEPCK-C gene transcription. An insulin responsive sequence (IRS) has been identified in the rat PEPCK-C promoter (-420 to -402) which coincides with the AF2 site. Phorbol esters regulate PEPCK-C gene expression in an insulin-mimetic fashion, use similar elements in the rat PEPCK-C promoter and act via the PI-3-kinase pathway. The AF2 site has been shown to be required for the PEPCK-C gene's response to glucocorticoids (5). It is thought that binding of an as yet unidentified protein to the IRS interferes with the binding of positive transcription factors and thus blocks the glucocorticoid effect in liver cells. The insulin signal transduction pathway that acts to regulate the PEPCK-C gene involves phosphoinositol 3-kinase (PI-3-kinase) but not the mitogen-activated kinase or the p70/p85 ribosomal S6 kinase pathways. Although studies of insulin-regulated genes have contributed enormously to the study of mechanisms of insulin action, no true consensus insulin response element has been defined, and the evidence gathered so far suggests that there is no such thing as a common cis-element which is used for insulin responses.

An excellent gene model for studying regulation of gene expression by insulin is the one for rat gene 33 (6). Rat gene 33 (g33) mRNA levels are elevated markedly by insulin, cAMP and glucocorticoids in rat liver (6), and in addition, by phorbol esters, glucocorticoids, insulin and plant lectins, retinoic acid, vanadium ions and calcium ionophores but not by cAMP, in rat H4IIE hepatoma cells (reviewed in ref. 7). Gene 33 functions as an immediate-early gene in regenerating liver, in mitogen-treated Reuber H-35 rat hepatoma, and Balb 3T3 mouse cells (8,9). This pattern of gene regulation suggests that g33 participates in the transition from quiescence to proliferation in many mitogen-treated cells, in addition to being involved in response to many extracellular agents.

Gene 33 is also under developmental control in the rat, since the gene is activated just prior to birth (10), followed by a further increase at birth. The $mRNA^{g33}$ is significantly increased by insulin, cAMP, and glucocorticoids in livers of fetuses treated in utero (11). Expression of this gene is not tissue specific (6, 8), since high levels of expression are present in liver and kidney, lower levels in heart, lung and testis in the rat. The mRNA turnover in liver is fast ($t_{1/2}$= 1 hr), and the rat gene has a potential to code for two polypeptides, the size of the translated products being about 42 and 50 kDa (12,13).

The exact function of g33 proteins is not known, but sequence analysis of the predicted amino acid sequence decoded from the $mRNA^{g33}$ sequences suggest soluble intracellular proteins (13,14). The deduced g33 protein sequence contains several regions that are similar to functional or regulatory domains of other known proteins. A human cDNA clone called *mig-6* has been recently isolated, whose predicted protein product has an 82.3% similarity to the rat g33 polypeptide (15). Haber et al. (16) demonstrated that human $mRNA^{g33}$ is expressed in liver and induced in fulminant hepatic failure. The strongest similarity to other protein sequences in the NCBI database is to the human non-receptor tyrosine kinase called ACK that inhibits the GTPase activity of $p21^{cdc42}$ (17). The similarity with the ACK kinases is highest in portions of the C-terminal half of both proteins, including a Class I SH3 domain binding site. The widespread and perhaps ubiquitous tissue distribution of g33 suggests that this gene may participate in a number of different responses perhaps as a member of a common signal transduction pathway.

In H4IIE rat hepatoma cells, the g33 response to insulin has been studied extensively, and has been shown to be specific (uses high affinity receptors) and rapid in serum-deprived cells. It was large and maximal in 1 hr (18,19). The effect of insulin on g33 expression in H4IIE cells has been compared to that of various insulin-mimetic agents. Insulin does not affect the stability of $mRNA^{g33}$ (18, 20), nor is insulin action completely inhibited by prior treatment with phorbol ester. Phorbol esters have insulin-like action on g33 induction (30-to 35-fold) that is synergistic with the action of insulin when very low concentrations of the tumor promoter are added before treatment with insulin. At submaximal concentrations of phorbol ester and at submaximal time of phorbol ester treatment, an additive effect of insulin and the tumor promoter was seen together (20). The ability of insulin to stimulate $mRNA^{g33}$ production was reduced by pretreatment with a phorbol ester (20), which suggests that PKC is necessary for insulin to exert its full effect on g33 transcription and cellular $mRNA^{g33}$ accumulation.

A portion of g33 5'-flanking DNA (-2600 to +27, and -480 to +27) has been analyzed for presence of cis-acting elements that may play a role in the regulation of gene 33 transcription, using chimeric plasmids in which expression of the chloramphenicol acetyl transferase (CAT) reporter gene was governed by the g33 5'-flanking DNA (21). In stable expression and *in vitro* transcription assays, expression of CAT was sensitive to insulin treatment of rat H4IIE cells.

In order to compare the regulation of gene 33 by insulin and phorbol esters with that of other well-studied genes, we studied the effect of insulin and PMA in two Chinese

Hamster ovary (CHO) cell lines: CHOwt and CHO-T cells. CHOwt express ~10^3 receptors/cell whereas CHO-T cells are stably transfected and overexpress ~5 X 10^6 human insulin receptors (hIR)/cell. These two CHO cell lines were chosen because they offered the opportunity to compare the effect of insulin on g33 expression in cells expressing very low or very high levels of the insulin receptor, to test the possibility of divergent or convergent pathways in insulin and phorbol ester signal transduction. Similar CHO cell lines have been used successfully by others to study the mechanisms of insulin action on other mitogen-inducible genes (c-fos, egr-1). We found that both insulin and phorbol esters cause elevations in mRNA^{g33} levels, but differences were observed in the g33 regulation in both CHO cell lines. Insulin appears to regulate mRNA^{g33} levels by mechanisms independent of the hIR in CHO wild type cells. Activation of conventional protein kinase C (PKCs) or PKC-dependent events do not appear to be involved in the insulin regulation of CHO g33 expression.

Materials and Methods

Materials. RNA molecular weight standards, porcine insulin, TRIzol reagent, agarose and actinomycin D were purchased from GIBCO/BRL Life Technologies. Phorbol 12- myristate 13-acetate (PMA), dimethylsulfoxide, diethylpyrocarbonate, formaldehyde and formamide were purchased from SIGMA. MOPS buffer and RNA gel-loading solution were purchased from Quality Biological.

Cell culture. The CHO cell lines were a generous gift from Dr. Richard A. Roth (Department of Pharmacology, Stanford University). Cells were maintained at 37° C in α-MEM supplemented with 8% fetal bovine serum (FBS), 2 mM glutamine, 100 μ/mL of penicillin/streptomycin and 5% CO_2. All media components were purchased from GIBCO/BRL.

Isolation of total cellular RNA and Northern Blot hybridizations. Total RNA was isolated using the TRIzol reagent, as recommended by the manufacturer. Aliquots of 25μg were electrophoresed in formaldehyde-containing gels with MOPS buffer as described (22). Ethidium bromide (1 μg) was added to each sample prior to loading. The gels were transferred to nylon membranes (Nytran N+, Amersham) and hybridized with ^{32}P-labeled probes (NEBlot kit, New England Biolabs), followed by autoradiography or phosphor imaging. For detection of the g33 mRNA, we used the 1.9 Kb HindIII/HindIII fragment from plasmid pTE201 (12) and autoradiography. To verify that the observed results were not a generalized response, but a gene-specific response, all Northern Blot membranes were deprobed and hybridized to a non-insulin-responsive probe, a chicken α-tubulin probe kindly provided by Dr. Leonard Jarett at the University of Pennsylvania School of Medicine (23). The α-tubulin hybridization signals were detected using a BIORAD GS-525 Molecular Imager. The amount of RNA loaded in each lane was assessed by densitometric scanning of the 28S rRNA band signal. The ethidium bromide and the g33 autoradiographic signals were analyzed in a GS-670 densitometer using the Molecular Analyst software.

Results

The size of the CHO mRNA^{g33} was determined to be 3.0 Kb, slightly smaller than the size of the rat hepatoma or liver mRNA^{g33}. Representative gels are shown in figures 1 and 2. Insulin (14 nM) stimulated the cytoplasmic accumulation of mRNA^{g33} in both CHO cell lines, compared to untreated cells. Stimulation was evident after

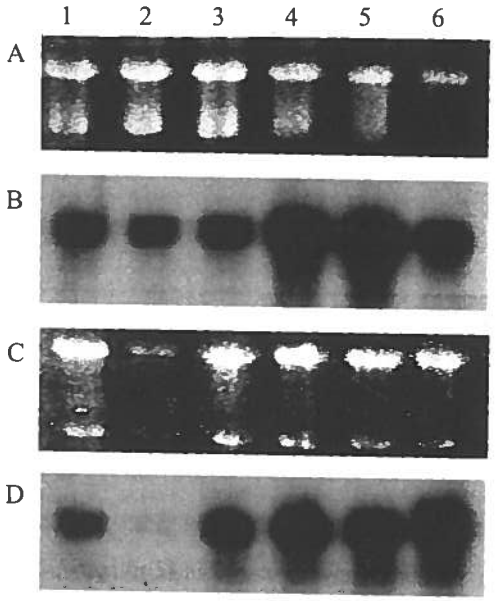

Figure 1. Effect of insulin on g33 mRNA levels in CHOwt and CHO-T cells. Cells were serum starved for 24 h and treated with 14 nM insulin for 0, 15 min, 30 min, 1h, 3h and 5 h. Total RNA was isolated and was analyzed by Northern blot hybridization with a ^{32}P-labeled g33 probe as described in the methods section.

A. RNA agarose gel electrophoresis of CHOwt samples; B. G33 Northern blot hybridization results for gel in A. C. RNA agarose gel electrophoresis of CHO-T samples; D. G33 Northern blot hybridization results for gel in C. Lane 1, control untreated; lane 2, 15 min insulin; lane 3, 30 min insulin; lane 4, 1h insulin; lane 5, 3 h insulin; lane 6, 5 h insulin.

30 minutes of treatment with the hormone and reached a maximum at 3 h (3.7 fold) in CHOwt and at 5 h (5-fold) in CHO-T cells (Fig. 1B and D, respectively and Table 1). In contrast, PMA stimulated mRNA^{g33} accumulation in 24 h serum-starved cells maximally at 3h (4-fold effect

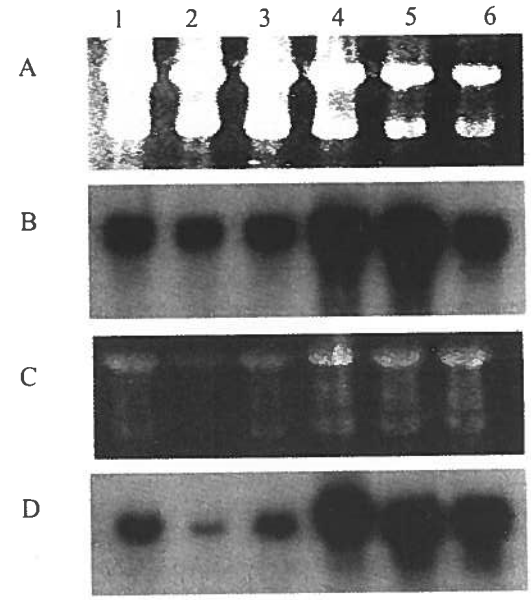

Figure 2. Effect of PMA on g33 mRNA levels in CHOwt and CHO-T cells. Cells were serum starved for 24 h and treated with PMA (1mg/mL) for 0, 15 min, 30 min, 1h, 3h and 5 h. Total RNA was isolated and was analyzed by Northern blot hybridization with the g33 probe.

A. RNA agarose gel electrophoresis of CHOwt samples; B. G33 Northern blot hybridization results for gel in A. C. RNA agarose gel electrophoresis of CHO-T samples; D. G33 Northern blot hybridization results for gel in C. Lane 1, control, untreated; lane 2, 15 min PMA; lane 3, 30 min PMA; lane 4, 1h PMA; lane 5, 3 h PMA; lane 6, 5 h PMA.

for both CHO cell lines, Fig. 2 and Table 1). In some sets of experiments the fold-induction by both insulin and PMA were of higher magnitude and maximum effects occurred at earlier times of treatment (Table 2). The control gene's expression (α-tubulin) was unaffected by insulin or PMA treatment in both cell lines (data not shown).

In order to establish whether both agents were stimulating the $mRNA^{g33}$ accumulation by similar mechanisms, simultaneous addition of both agents and desensitization experiments were carried out. Addition of both insulin and PMA simultaneously to serum starved CHO cells caused an additive effect at 5 hours of treatment in both cell lines (Fig. 3, Table 2), but not at lower treatment times (1-3 h). When cells were pretreated for 24 h with insulin followed by an additional 1h treatment with insulin the $mRNA^{g33}$ levels increased to a slightly lower extent than in non-desensitized cells (2.6-fold vs. 2.9 -fold on CHOwt cells and 2.5 vs. 4.0-fold increase in CHO-T cells over the control, Fig. 4). When the 24-h insulin pretreatment was followed by a 1-h treatment with PMA, $mRNA^{g33}$ levels were increased to a different extent than in non-desensitized cells (CHOwt, 3.2-fold vs. 2.4-fold increase over control and for CHO-T, 2.7 vs. 3.7-fold increase over controls, Fig. 4). When CHO cells were serum-starved and pre-incubated with PMA (1 μg/mL) for 24 h, followed by subsequent addition of PMA (1 μg/mL) or insulin 5 x 10^{-9} M) for 1 h, the elevations in $mRNA^{g33}$ levels were smaller than in non-desensitized

Table 1. Effect of Insulin and PMA on mRNAg33 Levels at Different Times of Treatment.

Treatment	Average fold effect* in cytoplasmic mRNAg33 levels in CHOwt cells	Average fold effect* in cytoplasmic mRNAg33 levels in CHO-T cells
Control, untreated	1.0	1.0
Insulin, 15 min	1.12	0.18
Insulin, 30 min	1.24	2.36
Insulin, 1h	2.90	4.02
Insulin, 3h	3.72	4.41
Insulin. 5h	2.32	5.09
PMA, 15 min	0.78	0.37
PMA, 30 min	1.10	1.12
PMA, 1h	2.39	3.76
PMA, 3h	4.04	4.22
PMA, 5h	3.18	3.41

* These values represent the average of two different experiments

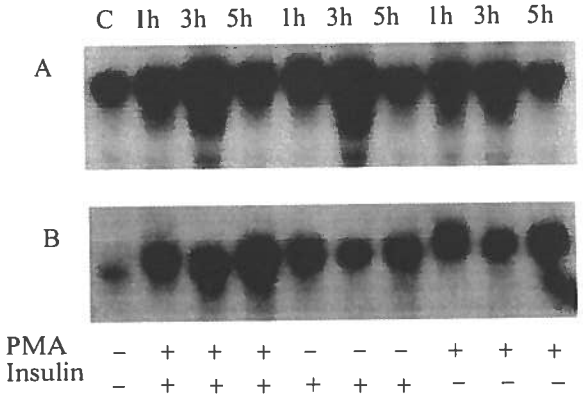

Figure 3. Effect of simultaneous treatment with PMA and insulin in CHOwt and CHO-T cells on g33 expression. Cells were serum starved for 24 h and treated with insulin (14 nM), PMA (1μg/mL) or a combination of the two agents at the designated concentrations for varying amounts of time. RNA was isolated, Northern blots prepared and membranes hybridized with a ^{32}P- labeled g33 probe. A: G33 Northern blot results for total RNA isolated from CHOwt cells ; B: G33 Northern blot results for total RNA isolated from CHO-T cells. (+) denotes addition of PMA and/or insulin as indicated in the figure; (-) denotes absence of the indicated agent.

Table 2. Interaction between Insulin and PMA on Gene 33 Expression in CHO Cells

Treatment	Average fold effect* in cytoplasmic mRNAg33 levels in CHOwt cells after insulin (14 nM) treatment	Average fold effect* in cytoplasmic mRNAg33 levels in CHO-T cells after PMA (1 µg/mL) treatment
Control, untreated	1.0	1.0
Insulin, 1h	4.24	8.4
Insulin, 3h	5.99	6.64
Insulin, 5h	2.62	6.64
PMA, 1h	3.58	10.58
PMA, 3h	4.36	7.3
PMA, 5h	1.77	8.5
Insulin + PMA, 1h	3.45	4.94
Insulin + PMA, 3h	6.82	8.81
Insulin + PMA, 5h	4.62	15.02

* These values represent the average of two different experiments

cells (CHOwt, 2.2-fold; CHO-T, 1.6-fold, Fig. 4).

The sensitivity of g33 to different doses of both insulin and PMA was also examined. Serum-starved cells were treated with various concentrations of insulin (10^{-11} to 10^{-7} M) for 1 h. Maximum effects were observed at an insulin concentration of 10^{-9} M in CHOwt cells, whereas for CHO-T cells, the optimum concentration was 10^{-11} M. Treatment of CHO-T with high concentrations of the hormone (10^{-8} to 10^{-7} M) reduced the $mRNA^{g33}$ levels (Fig. 5). When cells were treated with PMA for 1 h (0.01 to 10 µg/mL), maximal effects were observed at [PMA]= 0.1 µg/mL for CHOwt (3.4-fold) and at [PMA]=0.01 µg/mL for CHO-T cells (4.0-fold). High concentrations of PMA (10 µg/mL) caused small increases in the $mRNA^{g33}$ levels in CHOwt and CHO-T cells (Fig. 6).

Since the observed insulin response was specific and sensitive in both cell lines, the effect of RNA synthesis inhibitors on the accumulation of $mRNA^{g33}$ was examined to determine if the insulin response was due to an increase in the stability of $mRNA^{g33}$. CHO cells were treated with insulin (14 nM) to increase $mRNA^{g33}$ levels, then actinomycin D added (5µg/mL in media supplemented with 1% serum) for various treatment times (0.25, 0.5, 1, 2 and 4 h). The $mRNA^{g33}$ half-life was determined by calculating the rate of $mRNA^{g33}$ decay. The rate of $mRNA^{g33}$ decay was not significantly altered in cells exposed to insulin or left untreated for both cell lines used

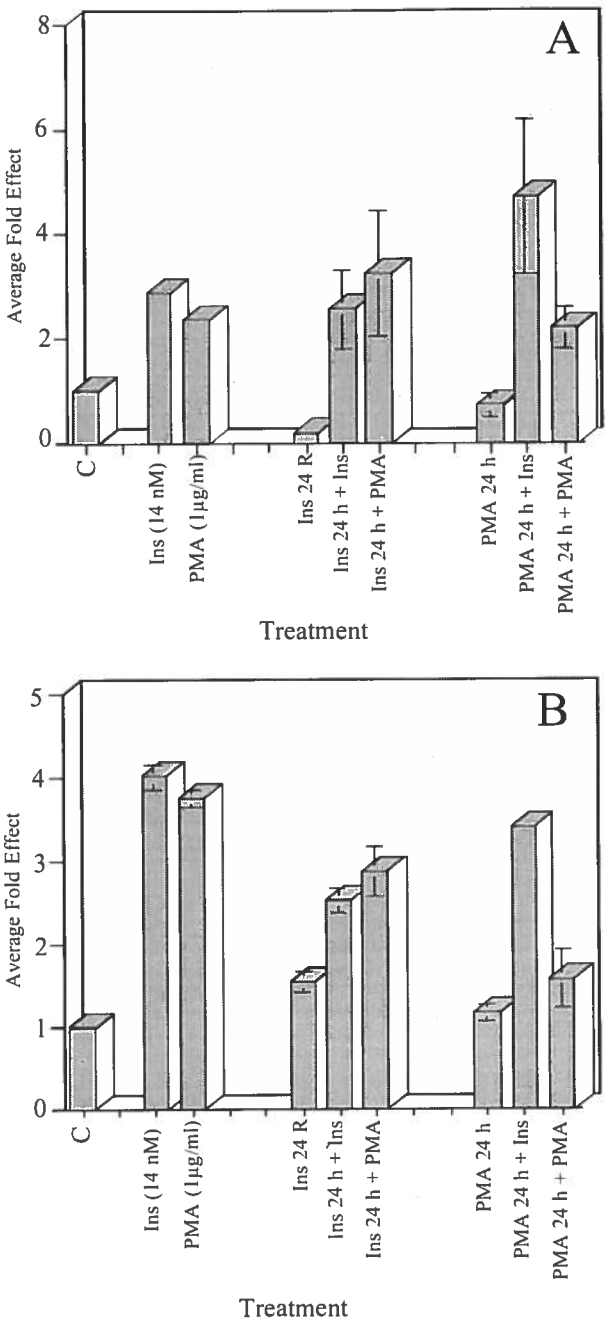

Figure 4. Effect of insulin desensitization and PMA pretreatment on g33 expression in CHO cells. Serum starved CHO cells were treated with insulin (5 x 10^{-7}M) or PMA (1µg/mL) for 24 h or 1 h or left untreated (control). Following the 24 h pretreatment either insulin (5 x 10^{-9}M) or PMA (1µg/mL) was added for 1 h. RNA was isolated, Northern blots prepared and membranes hybridized with a ^{32}P -labeled g33 probe. **A:** G33 Northern blot results for total RNA isolated from CHOwt cells; **B:** G33 Northern blot results for total RNA isolated from CHO-T cells. The intensities of the $mRNA^{g33}$ signals relative to the large rRNA bands were determined by densitometric scanning.

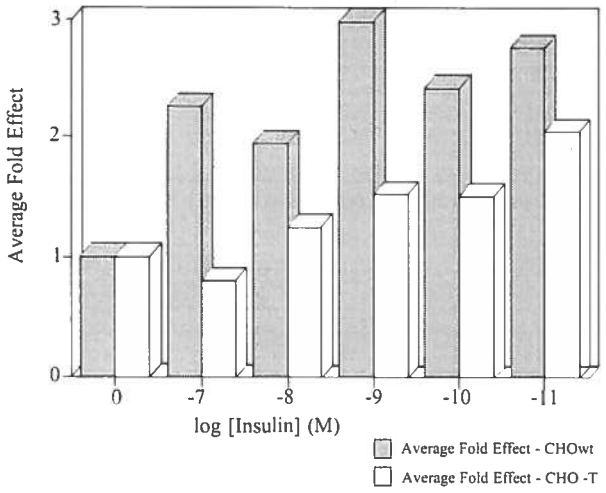

Figure 5. Effect of different concentrations of insulin on mRNA^{g33} levels in CHO cell lines. Serum-starved CHOwt (A) and CHO-T cells were treated with insulin for 1 h at the indicated molar concentrations. Total RNA was isolated and analyzed by Northern blot hybridizations with a ^{32}P-labeled g33 probe. The relative amounts of mRNA^{g33} were determined by densitometric tracing of the autoradiographs. The histograms represent the average fold effects determined in two different experiments.

in this study (Fig. 7, Table 3). In CHOwt cells, the mRNA^{g33} half life was 97 min in control cells and 109 min in insulin-treated (14 nM, 3 h) cells, whereas in CHO-T cells, the mRNA^{g33} half life was 51 min in control cells

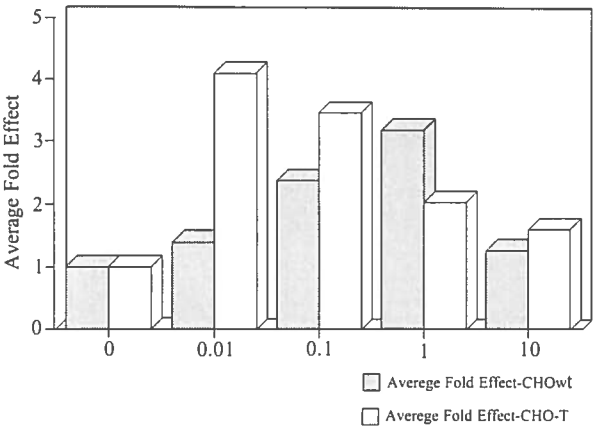

Figure 6. Effect of different concentrations of PMA on mRNA^{g33} levels in CHO cell lines. Serum-starved CHOwt (A) and CHO-T cells were treated with PMA for 1 h at the indicated concentrations as described in Materials and Methods. Total RNA was isolated and analyzed by Northern blot hybridizations with a ^{32}P-labeled g33 probe. The intensities of the mRNA^{g33} signals relative to the large rRNA bands were determined by densitometric scanning.
The histograms represent the average fold effects determined in two different experiments.

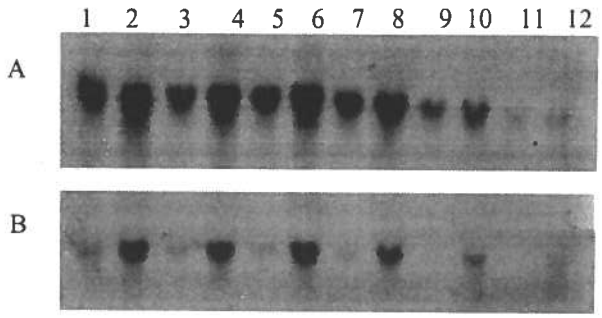

Figure 7. Northern blot analysis of the effect of insulin on the half-life ($t_{1/2}$) of mRNA^{g33} in CHO cell lines. CHO cells were serum starved for 24 h and then treated with insulin (14 nM) in media supplemented with 1% FBS for 3h or left untreated. After 3 h of insulin treatment, the media was removed and replaced with media supplemented with 1% FBS and Actinomycin D (5µg/mL) and total cellular RNA isolated at 0, 0.25, 0.5, 1, 2, and 4 h after addition of actinomycin D. Control samples received no insulin treatment. Northern blots of total RNA from CHOwt (A) and CHO-T (B) cells were hybridized to ^{32}P- labeled g33 and α-tubulin probes, and hybridization signals analyzed by phosphor imaging as described in the methods section. Even lanes represent control samples, odd lanes represent insulin-treated samples. Lanes 1-2, 0 time; Lanes 3- 4, 0.25 h; Lanes 5- 6, 0.5 h; Lanes 7-8, 1 h; Lanes 9-10, 2 h; and Lanes 11-12, 4h after addition of Actinomycin D.

Table 3. Effect of Insulin on the t1/2 of mRNAg33

Treatment	t1/2 in minutes
CHOwt	
Actinomycin D	109
Insulin + Actinomycin D	97
CHO-T	
Actinomycin D	51
Insulin + Actinomycin D	61

and 61 min in insulin-treated cells. These results suggest that the observed insulin effects do not alter the mRNA^{g33} stability significantly, and most probably affect a step before the appearance of the mature mRNA in the cytoplasm.

Discussion

Previous studies by Messina and co-workers have shown that insulin induces transcription and cytoplasmic accumulation of mRNA^{g33} in a time- and dose-dependent manner in rat hepatoma cells (18). Insulin exerts its effects at the level of transcription and post-transcription events, since the increases observed in the steady-state levels of mRNA^{g33} were of higher magnitude than those observed in the rate of transcription. Desensitization experiments did not abolish completely the insulin or PMA effects on

mRNA^{g33} levels in H4IIE cells, suggesting that both agents induce g33 mRNA accumulation at least in part by different pathways.

The results of the present studies in two CHO cell lines resemble the previous observations made in H4IIE cells to some extent. The increases in mRNA^{g33} levels in the two CHO cell lines are of smaller magnitude than that detected in rat hepatoma cells. It is remarkable that CHOwt cells, which express ~10^3 insulin receptors/cell are capable of responding to insulin in a similar manner (level of fold-induction and optimal time of response to 14 nM insulin) as CHO-T (which express ~10^6 hIR/cell) cells do. The main differences between these two cell lines lie in the optimal dose to observe maximum effects (10^{-11} M in CHO-T cells vs. 10^{-9} M in CHOwt cells) which is lower in the CHO-T cell line that expresses the highest number of IRs, and in the mRNA^{g33} half-life (about 1 h in CHO-T cells and ~95 min in CHOwt cells). The mRNA^{g33} half-life in CHO-T cells is very similar to that observed in rat liver cells. Insulin did not significantly alter the half-life of the mRNA^{g33}, suggesting that the observed effects are due to transcriptional events or other postranscriptional events independent of mRNA stability. Insulin's effects on mRNA^{g33} levels resemble those observed previously (23) in egr-1 gene regulation, which appear to be independent of the tyrosine phosphorylation of the IR and IRS-1. Since the CHOwt cell line does have a relatively small amount of functional Chinese hamster insulin receptors, the role they may have in the observed CHOwt insulin response warrants further studies, which are currently underway in our laboratory.

Recent studies (23) have shown that insulin regulates expression of *c-fos* and *egr-1* by two different mechanisms. Insulin was able to stimulate *c-fos* expression only in CHO_{HIRc} cells, which overexpress wild type human insulin receptors. Insulin did not increase the level of c-fos mRNA in the parent cells (CHO_{NEO}) and cells expressing ATP-binding site-mutated human insulin receptors (CHO_{A1018K}). In contrast, insulin was able to increase *egr-1* gene expression after 1 h of treatment (2.4-fold in CHO_{NEO} and 4.3-fold in CHO_{HIRc} cells) in CHO cells, even in cells lacking normal human insulin receptors, even when phosphorylation of IRS-1 was only detectable in CHO_{HIRc} cells. Other studies have shown that the abundance of receptors and the relative amounts of IRS-1 and other insulin receptor substrates are critical for the cellular sensitivity to insulin (24, 25).

PMA has been shown to cause large elevations of cytoplasmic mRNA^{g33} levels in rat H4IIE cells (30- to 35-fold increase in mRNA^{g33} by 1-2 h), which can be accounted for solely by its effect on the gene's rate of transcription since PMA doesn't alter the mRNA^{g33} half life (20, 26). PMA is also able to stimulate mRNA^{g33} accumulation in the two CHO cell lines tested, to a similar extent as that seen for insulin. The PMA response in CHO cells is reminiscent of the effects already demonstrated in rat H4IIE cells, although the increase in mRNA^{g33} levels is much lower than in rat hepatoma cells. PMA pretreatment failed to abolish the insulin response in both cells lines, but reduced the effect of a second PMA treatment, suggesting that the two agents act by using different pathways. The fact that the effect of both agents when added simultaneously was additive at 5 h of treatment also suggests that they act by different pathways.

The mechanisms by which insulin regulates gene transcription and other cellular functions are not completely clear, but there have been several reports that the activation of protein kinase C (PKC) activity is involved in mediating some of the hormone's actions (25). Jacobs and co-workers reported that PMA and insulin enhanced the IR receptor phosphorylation when added simultaneously (27). Messina and co-workers studied the effects of PMA and insulin together on mRNA^{g33} levels in H4IIE cells, but they didn't add both agents simultaneously (26). Additive effects were only observed at sub-maximal times of PMA treatment.

To explore the role of PKC in the insulin induction process, we performed pretreatment studies where we pre-incubated cells with insulin or phorbol esters and added insulin or phorbol ester subsequently to determine whether g33 was capable of being stimulated after the pre-incubation step. The pretreatment of serum-starved cells with high concentrations of insulin for 24 h resulted in a decrease in mRNA^{g33} levels. When insulin-desensitized CHOwt cells were exposed to insulin they were still able to induce g33 expression to a similar extent than insulin-treated control cells. These results suggest that insulin's ability to stimulate g33 expression is probably independent of the IR since pretreatment with insulin causes down regulation of the IR. In these insulin-desensitized cells, the ability of PMA to increase cytoplasmic mRNA^{g33} levels was not decreased (compared with the effect of treatment with 1 μg/mL PMA for 1h) in control cells (not pretreated with insulin).

Furthermore, the ability of insulin to increase mRNA^{g33} levels is not decreased in PMA-pretreated CHOwt cells. These data suggest that activation of conventional PKCs or PKC-dependent events are not involved in the regulation of g33 by insulin. These results were consistent with other studies, which indicate that the conventional and novel PKC species play little or no role in the action of insulin to stimulate transcription of genes like *c-fos* (3). We are unable to explain at this time the response of PMA-pretreated CHOwt cells to further PMA addition.

This effect has to be studied further, by using other phorbol esters or varying the duration of PMA pretreatment in CHO cell lines or testing for down-regulation of specific PKC isoforms.

Previous studies showed that insulin pretreatment of H4IIE hepatoma cells prevented the insulin stimulation of g33 transcription and the corresponding increase in $mRNA^{g33}$ levels. When insulin-desensitized cells were exposed to phorbol esters, the induction of g33 is decreased (7). One of the three intracellular consequences of insulin-desensitization may be an alteration in PKC-dependent events, since insulin desensitization abolished insulin's induction and impaired phorbol esters induction of g33 expression (7). PKC-deficient H4IIE cells lost their responsiveness to phorbol ester induction of g33, and the ability to increase transcription and $mRNA^{g33}$ levels was decreased, but not abolished (26).

The regulation of g33 by insulin in CHO-T cells was reminiscent of insulin's effects in H4IIE cells, in contrast with the CHOwt cells results. The prolonged exposure of CHO-T cells to insulin resulted in a reduction of insulin's ability to induce $mRNA^{g33}$ accumulation but did not abolish the insulin effect. Insulin appeared to stimulate g33 expression mainly through an IR-dependent pathway in CHO-T cells. In a published study where similar cell lines were used, IR and IRS-1 phosphorylation were detected in insulin-treated CHO_{HIRc} cells overexpressing wild type human IR, but barely detected in CHO_{NEO} cells (23). The fact that insulin-desensitized CHO-T cells were still able to respond to insulin in elevating $mRNA^{g33}$ levels indicates that a compensatory signal transduction pathway existed. This insulin pretreatment also caused a decreased responsiveness to phorbol esters. Thus, insulin desensitization may be affecting the activity of PKC or a substrate (s) of PKC.

PMA-pretreated CHO-T cells lost their ability to increase g33 expression in response to further PMA addition, as expected. The ability of insulin to increase $mRNA^{g33}$ levels is decreased but not abolished in PMA-pretreated CHO-T cells compared to PMA effects observed in control cells. This is evidence for a post-PKC effect since PMA directly activates PKC, bypassing the IR. Insulin desensitization of CHO-T cells, as in H4IIE cells, decreased PMA's induction of g33 expression, suggesting that insulin or its receptor may be involved in PKC activation or an alteration in PKC-dependent events.

In summary, both insulin and the phorbol ester PMA cause elevations of $mRNA^{g33}$ levels in two CHO cell lines. Insulin regulated g33 expression in the two different CHO cell lines apparently by different mechanisms. Overexpression of IR promoted the regulation of g33 expression mainly by the IR-mediated signal transduction pathway. Insulin appeared to regulate g33 expression in CHOwt cells by an insulin receptor phosphorylation-independent pathway. It is possible that different cell types have different signaling pathways and that the response to a hormone may not always be through the same cellular mechanism(s) (23). The cell culture conditions (such as culture passage number) may influence the extent and optimum time of the observed enhancement of gene 33 expression. The variability in the estimation of the enhancement of $mRNA^{g33}$ levels by insulin and PMA has been seen in other studies of g33 expression in H4IIE cells (7). The current study presents another example of the regulation of gene 33 expression by insulin and PMA. Our results provide support for distinct insulin-signaling pathways in the regulation of g33 expression, which may involve PKC- dependent events or PKC activation.

Resumen

El gen 33 (g33) de ratas se expresa en multiples tejidos. Su expresión es regulada por insulina y varios agentes tales como glucocorticoides, ésteres de forbol y lectinas en células de hepatoma de rata. La regulación del gen 33 por insulina y un éster de forbol se examinó en dos líneas celulares de ovario de güimo chino (CHO, por sus siglas en inglés), CHO-T sobreexpresan receptores de insulina humanos (hIR, por sus siglas en inglés) y cepas salvajes de células CHO, CHOwt. Estas líneas celulares se utilizaron para determinar como la expresión del hIR influencia la capacidad del g33 de responder a insulina y al acetato de miristato de forbol (PMA, por sus siglas en inglés). El tratamiento de células CHOwt y CHO-T con insulina aumentó los niveles de $mRNA^{g33}$ de 3-4 veces, siendo el efecto máximo luego de tres horas de tratamiento. El PMA causó un efecto similar en los niveles de $mRNA^{g33}$ de las células CHOwt y CHO-T luego de tres horas de tratamiento. Además, los efectos de insulina y PMA fueron aditivos solamente en células CHO-T. Células CHO-T pretratadas con insulina o PMA fueron capaces de responder a ambos agentes, pero la elevación en los niveles de $mRNA^{g33}$ fueron submáximos. En contraste, las células CHOwt pretratadas con insulina o PMA, el g33 respondió al máximo. Estos resultados sugieren que insulina y los ésteres de forbol actúan a través de diferentes mecanismos de traducción de señales en células CHOwt. Además, la capacidad de insulina de estimular la expresión del g33 en células CHOwt sugiere que este efecto de insulina es independiente de su receptor. Hay diferencias marcadas en el patrón de regulación del g33 por insulina y PMA en células de hepatoma de rata y las dos líneas celulares estudiadas.

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References

1. Waters SB, Pessin, JE. Insulin receptor substrate 1 and 2 (IRS1 and IRS2): what a tangled web we weave. Trends Cell Biol 1996; 6:1.
2. Ish-Shalom D, Christoffersen CT, Vorwerk P, Sacerdoti-Sierra N, Shymko RM, Naor D, De Meyts P. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. Diabetologia 1997;40 Suppl 2:S25.
3. Blackshear PJ. The role (or lack thereof) of Protein kinase C in insulin action. In: Draznin B, LeRoith D, and Eds. Molecular Biology of Diabetes II, Totowa, New Jersey, Humana Press; 1994. p. 229.
4. Hanson RW, Reshef L. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annual Rev Biochem 1997; 65:581.
5. O'Brien RM, Lucas PC, Forest CD, Magnuson MA, Granner DK. Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. Science 1990; 249:533.
6. Lee K-L, Isham KR, Stringfellow L, Rothrock R, Kenney FT. Molecular cloning of cDNAs cognate to genes sensitive to hormonal control in rat liver. J Biol Chem 1985;260:16433.
7. Messina, JL. Regulation of gene 33 expression. In: Draznin B, LeRoith D, and Eds. Molecular Biology of Diabetes II, Totowa, New Jersey, Humana Press; 1994. p. 263.
8. Mohn KL, Laz TM, Melby AE, Taub, R. Immediate-early gene expression differs between regenerating liver, insulin-stimulated H-35 cells, and mitogen-stimulated Balb/c 3T3 cells. J Biol Chem 1990;265:21914.
9. Mohn KL, Laz TM, Hsu J-C, Melby AE, Bravo R, Taub R. The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: Comparison with serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. Mol Cell Biol 1991;11:381.
10. Rothrock R, Lee K-L, Isham KR, Johnson AC, Kenney, FT. Different mechanisms controls developmental activation of transcription of genes subject to identical hormonal regulation in adult liver. Biochem Biophys Res Commun 1987;144:1182.
11. Johnson AC, Lee K-L, Isham KR, Kenney FT. Gene-specific acquisition of hormonal responsiveness in rat liver during development. J Cell Biochem 1988;37:243.
12. Tindal MH, Lee K-L, Isham KR, Cadilla C, Kenney, FT. Structure of a multihormonally regulated rat gene. Gene 1988;71:413.
13. Lee K-L, Makkinje A, Ch'ang L-Y, Kenney FT. Molecular cloning and analysis of full-length cDNAs cognate to a rat gene under multihormonal control. Arch Biochem Biophys 1989;269:106.
14. Chrapkiewicz NB, Davis CM, Chu DT, Caldwell CM, Granner DK. Rat gene 33: analysis of its structure, messenger RNA and basal promoter activity. Nucleic Acids Res 1989;17:6651.
15. Wick M, Bürger C, Funk M, Müller R. Identification of a novel mitogen-inducible gene (*mig-6*): regulation during G_1 progression and differentiation. Exp. Cell Res 1995;219:527.
16. Haber BA, Mohn KL, Diamond RH, Taub R. Induction patterns of 70 genes during nine days after hepatectomy define the temporal course of liver regeneration. J Clin Invest 1993;91:1319.
17. Manser E, Leung T, Salihuddin H, Tan L, Lim L. A non-receptor tyrosine kinase that inhibits the GTPase activity of $p21^{cdc42}$. Nature 1993;363:364.
18. Messina JL, Hamlin J, Larner J. Effects of insulin alone on the accumulation of a specific mRNA in rat hepatoma cells. J Biol Chem 1985;260:16418.
19. Chu DT W, Davis CM, Chrapkiewicz NB Granner DK. Reciprocal regulation of gene transcription by insulin. ibid. 1988;263:13007.
20. Messina JL, Hamlin J, Larner J. Positive interaction between insulin and phorbol esters on the regulation of a specific messenger ribonucleic acid in rat hepatoma cells. Endocrinology 1987; 121:1227.
21. Cadilla CL, Isham KR, Lee K-L, Ch'ang L-Y, Johnson AC, Kenney FT. Insulin increases transcription of rat gene 33 through cis-acting elements in 5'-flanking DNA. Gene 1992;118:223.
22. Sambrook J, Fritsch EF and Maniatis T. Molecular Cloning, a laboratory manual, Second edition, Cold Spring Harbor, NY. Cold Spring Harbor Laboratory Press; 1989. p.7.43.
23. Harada S, Smith RM, Smith JA, Shah N, Hu D-Q, Jarett L. Insulin-induced egr-1 expression in Chinese Hamster ovary cells is insulin receptor and insulin receptor substrate-1 phosphorylation-independent: evidence of an alternative signal transduction pathway. J Biol Chem 1995;270:26632.
24. Maggi D, Laurino C, Andraghetti G, Cordea R. The overexpression of insulin receptor makes CHO cells resistant to the action of IGF-1: role of IRS-1. Biochem Biophys Res Commun 1994;205:12, 693.
25. Yamauchi K, Pessin JE. Enhancement of insulin signaling by insulin receptor substrate 1 is cell context dependent. Mol Cell Biol 1994;14:4427.
26. Weinstock RS, Messina, JL. Transcriptional regulation of a rat hepatoma gene by insulin and protein kinase C. Endocrinology 1988;123:366.
27. Jacobs S, Sahyoun NE, Saltiel AR, Cuatrecasas P. Phorbol esters stimulate the phosphorylation of receptor for insulin and somatedin C. Proc Natl Acad Sci USA 1983;80:6211.
28. Ways DK, Cook PP, Webter C, Parker, PJ. Effect of phorbol esters on protein kinase C-zeta. J Biol Chem 1992;267:4799.