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# Insulin-Induced Gene 33 mRNA Expression in Chinese Hamster Ovary Cells Is Insulin Receptor Dependent

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**Abstract** Gene 33 (g33) is a non-tissue-specific gene regulated in rat liver and hepatoma cells by insulin and other agents. It is thought to participate in the transition from quiescence to proliferation in mitogen-treated cells. The mechanism(s) by which insulin exerts its action on g33 are not totally understood; it is unclear whether a functional insulin receptor is required for this action. In this study, we evaluate the mechanism for insulin induction of g33 mRNA in Chinese hamster ovary (CHO) cells transfected with the neomycin-resistant plasmid (CHONeoB), human insulin receptor (CHONewIRa), and a kinase-defective insulin receptor mutated at the ATP-binding site (CHOK1018A). Transfected cells had higher levels of insulin binding than that of CHONeoB cells; insulin-induced phosphorylation of the insulin receptor and its intracellular substrates were impaired in CHOK1018A cells. Maximal insulin induction of $mRNA^{g33}$ occurred 3 h after hormonal exposure in all cell lines. The degree of insulin stimulation of g33 mRNA levels was four- to sixfold higher in CHONewIRa than in CHONeoB or CHOK1018A cells, which had minimal levels of insulin-stimulated g33 mRNA levels. Half-maximal stimulation of g33 mRNA levels was observed at 0.06 ± 0.01 nM in CHONewIRa cells, consistent with insulin interaction with its own receptor. Wortmannin, an inhibitor of phosphatidyl inositol 3-kinase (PI3K), had some effects on insulin stimulation of g33 mRNA in CHO NewIRa cells. PD98059, an inhibitor of mitogen-activated kinase kinase (MAPKK), and rapamycin, a p70 S6 kinase inhibitor, had minimal effect on insulin stimulation of g33 mRNA in all cells tested. By contrast, hydroxy-2-naphthalenylmethyl)phosphonic acid triacetoxymethyl ester (HNMPA$(AM)_3$, a selective inhibitor of the insulin receptor tyrosine kinase, caused complete inhibition of insulin stimulation of g33 mRNA levels. These data indicate that the insulin receptor with intact kinase activity is required for insulin stimulation of g33 mRNA levels. They also suggest that AKT, a PI 3-kinase downstream effector molecule, could mediate insulin stimulation of g33 mRNA. The mechanism(s) of insulin regulation of g33 expression downstream of receptor do not seem to rely entirely on the classic insulin receptor transduction pathway, as a minor effect was observed upon inhibition of MAPKK, suggesting that multiple pathways may be involved. J. Cell. Biochem. 77:432–444, 2000. 

**Key words:** insulin; CHO cells; gene 33 mRNA; insulin receptor

Insulin initiates its action by binding to a specific cell surface receptor that is ubiquitously distributed. The insulin receptor possesses an intrinsic tyrosine kinase activity that

Abbreviations used: g33, gene 33; *mig*-6, mitogen-activated gene 6; PI 3-kinase, phosphatidylinositol 3′-kinase; CHO, Chinese hamster ovary; IR, insulin receptor; hIR, human insulin receptor; IRS-1, insulin receptor substrate-1; p70 S6, p70/p85 ribosomal protein kinase; MAPKK, mitogen-activated protein kinase kinase; HNMPA, (hydroxy-2-naphthalenylmethyl)phosphonic acid; HNMPA$(AM)_3$, (hydroxy-2-naphthalenylmethyl)phosphonic acid triacetoxy methyl ester; FBS, fetal bovine serum; WGA, wheat germ agglutinin, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS; 3-(N-morpholino)propanesulfonic acid; EBSS, Earle's balance salt solution; HEPES, N-[2-hydroxyethylpiperazine-N′-2 ethanesulfonic acid].


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is stimulated by insulin binding [Ullrich et al., 1985]. Insulin regulates the expression of many genes in adipose tissue, skeletal and cardiac muscle, and liver [O'Brien et al., 1996]. Examples include the gene encoding for phosphoenolpyruvate carboxykinase [Andreone et al., 1982], tyrosine aminotransferase [Lee et al., 1986], glyceraldehyde 3-phosphate dehydrogenase [Alexander et al., 1985], hexokinase II [Printz et al., 1993], and the protooncogenes c-*fos* [Stumpo et al., 1986] and *egr-1* [Sukhatme et al., 1987]. Interestingly, transcription is repressed in some instances (e.g., phosphoenolpyruvate carboxykinase, growth hormone) and is increased in others, an aspect of insulin control of gene expression that is not completely understood.

The gene 33 (g33) cDNA was originally isolated by differential screening of a library prepared from hydrocortisone-induced rat liver poly($A^+$) mRNA [Lee et al., 1985]. The corresponding gene spans 13,550 bp encoding 2,970 bp of exon sequences interrupted by three introns [Tindal et al., 1988; Lee et al., 1989; Chrapkiewicz et al., 1989]. Its expression in rat liver is regulated independently by different agents, such as glucocorticoids, insulin, cyclic adenosine monophosphate (cAMP), phorbol esters, plant lectins, calcium ionophores, and retinoic acid [Messina, 1994]. All these compounds increase the levels of g33 mRNA and presumably elevate the corresponding protein levels. Gene 33 is also under developmental control in rat liver, as it is activated just before birth, followed by a further increase at birth [Rothrock et al., 1987]. Expression of g33 is widespread with tissue-specific variations [Mohn et al., 1990], with high levels in liver and kidney and lower levels in heart, lung, and testis in rats [Lee et al., 1985; Mohn et al., 1990]. In humans, the gene is expressed in the liver; its expression increases after partial hepatectomy [Harber et al., 1993]. In liver, the mRNA turnover is fast (half-life [$t_{1/2}$] = 1 h) [Lee et al., 1985]. Gene 33 has the potential to code for two unique proteins of 459 and 383 amino acids, with translated products of about 42 and 49 kDa, which appear to be the result of alternative usage of splice sites [Lee et al., 1985, 1989].

Results reported by different research groups [Mohn et al., 1990, 1991; Harber et al., 1993; Almendral et al., 1988; Wick et al., 1995] studying immediate-early genes suggest that g33 participates in the transition from quiescence to proliferation in mitogen-treated cells. 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 [Mohn et al., 1990, 1991; Harber et al., 1993; Almendral et al., 1988; Wick et al., 1995], but evidence for this hypothesis requires further testing. Previous studies had been designed to investigate the effects of insulin alone on the $mRNA^{g33}$ levels [Messina, 1985, 1994] in hepatoma cells. In 24-h serum-deprived rat liver hepatoma cells (H4IIEC3), insulin induced a threefold increase in the levels of $mRNA^{g33}$ within 30 min, with a maximum (10-fold) increase observed at 1 h. The effects of insulin were evident at concentrations as low as $10^{-12}$ M and were maximal at $0.5–1 \times 10^{-8}$ M. Granner and coworkers [Chu et al., 1988] observed a sevenfold increase of g33 mRNA upon insulin stimulation in H4IIE cells. An attempt to identify the signaling processes responsible for the selective effects of insulin on the expression of gene 33 in H4 hepatoma cells was performed by Yang and Dickson [1995a, b]. These investigators reported that wortmannin [Arcaro and Wymann, 1993] abolished the expression of $mRNA^{g33}$, suggesting that phosphatidyl inositol 3-kinase is required for gene 33 induction by insulin. There are no studies of insulin stimulation of g33 mRNA levels in Chinese hamster ovary (CHO) cells expressing normal or mutant insulin receptors.

In this study, the effects of insulin on the endogenous g33 $mRNA^{g33}$ levels were compared in CHO cells stably-transfected with the normal (CHONewIRa) [Longo et al., 1992] and a tyrosine kinase mutant insulin receptor mutant (CHOK1018A) [Chou et al., 1987], and control CHONeoB cells, which were stably transfected with the neomycin-resistance gene. We found insulin stimulation of g33 mRNA levels to be markedly reduced in cells expressing the kinase-defective insulin receptor; in addition, hydroxy-2-naphthalenylmethyl phosphonic acid triacetoxymethyl ester ($HNMPA(AM)_3$), a selective inhibitor of the insulin receptor tyrosine kinase [Saperstein et al., 1989], prevented insulin stimulation of g33 mRNA levels. It also abolished insulin-induced tyrosine phosphorylation of IR and pp185. Wortmannin, a phosphatidylinositol 3-kinase inhibitor [Arcaro and

Wymann, 1993], showed some inhibitory effects. PD98059, a specific inhibitor of the mitogen-activated protein kinase kinase (MAPKK) [Alessi et al., 1995], led to minimal reduction of insulin effects, and rapamycin, an inhibitor of the p70 S6 kinase [Chung et al., 1992], caused no significant reduction in the effects of insulin on the mRNA$^{g33}$ levels.

## MATERIALS AND METHODS

### Materials

The plasmids containing the cDNA for α-tubulin was kindly provided by Dr. L. Jarret (University of Pennsylvania). Gene 33 pTE201 plasmid was previously generated by Tindal et al. [1988]. The [α-$^{32}$P]dCTP and enhanced chemiluminescent (ECL) reagent kit were obtained from Amersham (Arlington Heights, IL). Tissue culture medium and fetal bovine serum (FBS) were obtained from Gibco-Life Technologies (Gaithersburg, MD); TRI Reagent from Molecular Research Center (Cincinnati, OH) was used for total RNA isolation. Mouse monoclonal antibody against phosphotyrosine (PY-20) and rabbit polyclonal antibody against IR β-subunit (IRβ C-19) were obtained from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively; pAKT rabbit polyclonal antibody from New England Biolabs (Beverly, MA); and Phospho-MAPK rabbit polyclonal antibody from New England Biolabs and AKT mouse monoclonal antibody from Transduction Laboratories. The polyclonal antibody against p31 S6 was kindly provided by M. Birnbaum (University of Pennsylvania). The anti-mouse IgG and anti-rabbit IgG peroxidase-linked secondary antibodies were purchased from Amersham. Porcine insulin was acquired from Biofluids (Rockville, MD), and the agarose-wheat germ agglutinnin (WGA) from Vector Laboratories (Burlingame, CA). PVDF membranes (Immobilon-P) were from Millipore (Bedford, MA).

All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA). The inhibitors were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA).

### Cell Culture and Treatments

Transfected CHO cell lines stably transfected with the neomycin-resistant plasmid (CHONeoB) and wild-type human insulin receptors (CHONewIRa) were previously generated [Longo et al., 1992]. CHO cells expressing an insulin receptor mutated in the ATP binding site, in which lysine 1018 was substituted by alanine (CHOK1018A), were generously provided by Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Cells were cultured in Ham's F-12 medium containing 6% (v/v) FBS, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and nonessential amino acids (0.1 mM) at 37°C, with 5% $CO_2$. At 80% confluency, cells were transferred to medium containing 1% FBS for 16–24 h before the experiments. CHO cells were incubated with Ham's F-12 containing 1% FBS and 14 nM insulin for the time-course experiments (1–5 h) and for 3 h with 0.014, 0.14, 1.4, 14, and 140 nM insulin for the dose-response experiments. Wortmannin, rapamycin, and PD98059 were added to the cells 15–30 min before insulin (140 nM) stimulation, whereas HNMPA(AM)$_3$ was added 1 h before insulin stimulation (140 nM).

### RNA Isolation and Northern Blot Analysis

Total RNA was extracted from cells, using TRI Reagent according to the manufacturer's protocol. Total RNA (30 μg) was separated through 1% denaturing agarose 2.2 M formaldehyde/1% MOPS gel electrophoresis [Sambrook et al., 1989] and capillary-transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH). Specific DNA probes for gene 33 and α-tubulin were labeled with [α-$^{32}$P]-dCTP by the random primer DNA labeling method, using a kit from New England Biolabs. Membranes were prehybridized in 50% formaldehyde, 5× SSPE, 2% SDS, 10× Denhart's, and 100 μg/ml sonicated salmon sperm DNA (Stratagene, La Jolla, CA) at 42°C for 4–6 h, then hybridized with the respective probes at 2–5 × $10^6$ cpm/ml for 18–24 h. The membranes were washed with 2× SSC/0.05% SDS for 10 min at room temperature, and twice for 10 min at 50°C with 0.1× SSC/0.1% SDS. The $^{32}$P-labeled hybridization signals were identified and quantified using a 525 Molecular PhosphorImager with the Molecular Analyst Software (Bio-Rad). Gene 33 mRNA expression

was normalized against that of the α-tubulin gene and reported as a ratio to the control samples.

### pp185 Phosphorylation and Immunoblots

Confluent CHO cell cultures were starved for 3 days in serum-free Ham's F-12 media before the addition of insulin (400 nM) for 25 min. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS), scraped, and lysed by four freeze-thaw cycles, using 1 ml of lysis buffer containing 50 mM Hepes (pH 7.8), 1% (v/v) Triton X-100, 2.5 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 2 μM pepstatin, 10 μM leupeptin, and 0.5 TIU aprotinin. The lysate was cleared by centrifugation for 10 min at 10,000*g* at 4°C. Supernatant protein concentration was determined using the Bradford reagent method [Bradford, 1976]. For detection of pp185, 100 μg of cell lysate was run on 7.5% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 1% gelatin-containing solution and reacted with anti-phosphotyrosine antibody, PY20 at a (1:1,000) dilution. Blots were then incubated with an anti-mouse IgG, peroxidase-linked secondary antibody at a (1:9,000) dilution, followed by enhanced chemiluminescence (ECL) detection, according to the manufacturer's instructions (Amersham), and analyzed by autoradiography or by phosphorimaging.

For detection of pAKT, AKT, pMAPK, and p31S6, 30 μg of total cell lysate was run on 10% SDS-PAGE, transferred to PVDF. Membranes were blocked for 1 h at RT using Super Block™ Blocking Buffer (Pierce, Rockford, IL) and reacted with the respective antibodies sequentially, pAKT (1:1,000), AKT (1:1,000), and pMAPK (1:1,000) prepared in T-TBS containing 5% purified BSA pH 7.4 and 0.02% sodium azide for 16 h, 1 h, and 16 h, respectively. Then washed 3 times with T-TBS at RT. The p31 S6 rabbit polyclonal Ab (1:5,000) was prepared in T-TBS containing 1% dried milk, pH 7.4, and 0.02% sodium azide was incubated for 1 h at RT. Membranes were then incubated with secondary antibodies (anti-rabbit Ig horseradish peroxidase [HRP]-linked (1:3,500) (Amersham) and anti-mouse Ig HRP-linked, 1:3,500) for 1 h and rinsed 3 times with T-TBS. For detection, we used Super Signal® West Pico Luminol/Enhancer Solution (Pierce, Rockford, IL), according to the manufacturer's recommendations and analyzed by autoradiography.

### Insulin Receptor Autophosphorylation

Insulin receptors were isolated as described previously [Freidenberg et al., 1985]. Equal amounts of partially purified insulin receptors from WGA-agarose columns were mixed with autophosphorylation buffer (500 mM HEPES (pH 7.4), 1 mg/ml BSA, with or without 2.2 μM insulin [Brennan and Lin, 1996]. Mixtures were incubated for 15 min at RT. The phosphorylation reaction was initiated by adding 1 mM ATP, 10 mM $MgCl_2$, and 2 mM $MnCl_2$, mixed and incubated for 15 min at RT [Brennan and Lin, 1996]. Reactions were terminated by boiling the samples in Laemmli SDS-PAGE buffer [Laemmli, 1970]. Proteins were resolved by 7.5% SDS-PAGE under reducing conditions (100 mM DTT) and transferred onto Inmobilon-P membrane. Membranes were blocked and reacted with the PY20 antibody as above. Blots were incubated with HRP-linked secondary antibody at a (1:8,500) dilution and analyzed as described above.

### Insulin Binding and Glucose Transport

Insulin binding experiments were performed as previously described [Longo et al., 1992]. Briefly, confluent cells were incubated overnight at 4°C in Earle's balanced salt solution (EBSS) containing $^{125}$I-insulin (0.2–0.5 ng/ml, 350–500 nCi/ng) in the presence of increasing concentrations of cold insulin. Nonspecific binding was measured by adding 5 μg/ml of cold insulin and subtracted from all points. After overnight incubation, cells were washed three times with ice-cold saline solution and the cells with bound insulin were dissolved in 1 N NaOH with 0.1% deoxycholic acid. The content of each well was transferred to a counting tube; radioactivity was determined in a gamma counter. After counting, protein content was measured in each tube with a modified Lowry procedure [Wang and Smith, 1975]. Binding was normalized for protein concentration and corrected for nonspecific binding and expressed in fmole of insulin per milligram of cellular proteins. Data were analyzed by nonlinear regression analysis according to a two-receptor model as described previously [Longo et al., 1989].

Glucose transport assays were performed as previously described [Longo et al., 1992].

**TABLE I. Insulin Binding of CHO Cells Expressing Normal and Mutant Human Insulin Receptor***

| Cell line | Insulin binding (fmol/mg cell protein) | Receptors/cell | Half-maximal inhibition (nM) |
|---|---|---|---|
| CHONeoB | 1.82 | 5,800 | 0.42 ± 0.09 |
| CHONewIRa | 35.84 | 93,000 | 0.46 ± 0.05 |
| CHOK1018A | 10.50 | 39,300 | 1.06 ± 0.09 |

*Insulin-binding experiments were performed as previously described [Longo et al., 1992]. Receptor number was determined by nonlinear regression analysis of data according to a two-receptor model [Longo et al., 1989]. Receptor affinity was estimated from the insulin concentration required for half-maximal inhibition of tracer binding.

Briefly, confluent cells were incubated for 60 min in Earle's balanced salt solution supplemented with 0.1% BSA and glucose 2 g/L without or with insulin (200 nM). Wortmannin (100 nM) was added 5 min before insulin. Cells were then washed twice with glucose-free EBSS and 3-O-methyl[$^3$H]-D-glucose (OMG, 1 mM, 4 μCi/ml) transport was measured for 20 s. The data represent averages ±SD of triplicates.

## RESULTS

### Insulin Receptors in CHO Cells

To characterize the CHO cells used in this study, we measured their capacity to bind insulin by incubating confluent cells with $^{125}$I-insulin and increasing concentrations of cold insulin. Nonlinear regression analyses of the data according to a two-receptor model estimated 93,200 receptors (high-affinity binding sites) per cell for CHONewIRa, followed by CHOK1018A (39,300 receptors/cell) and CHONeoB with 5,800 receptors/cell (Table I). Half-maximal inhibition of insulin binding was observed at 0.42 ± 0.09 nM for CHONeoB cells, 0.46 ± 0.05 nM for CHONewIRa, and 1.06 ± 0.09 nM for CHOK1018A cells, respectively. To characterize receptor autophosphorylation, insulin receptors were partially purified by WGA chromatography and incubated in the presence or absence of insulin with ATP. Insulin receptors were then run on SDS-PAGE, blotted onto membranes, and detected using the PY20 monoclonal antibody directed against the phosphotyrosine residues.

Overexpression of the wild-type insulin receptor increased both basal receptor autophosphorylation and the insulin response (Fig. 1A) (CHONewIRa cells) as compared with preparations derived from CHONeoB cells that required overnight exposure to a chemiluminescence screen, as compared with a 15-min exposure, to evidence insulin receptor phosphorylation (Fig. 1B). As expected, CHOK1018A exhibited the lowest degree of insulin receptor phosphorylation in both the absence and the presence of insulin (Fig. 1A),

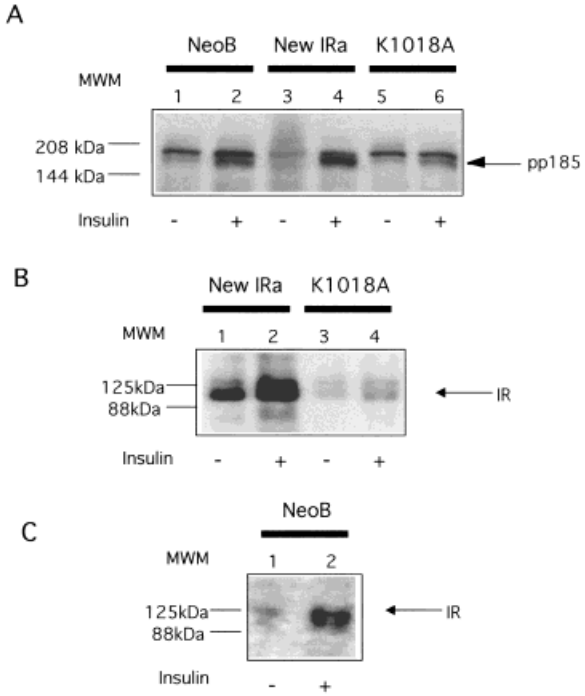


**Fig. 1.** Effects of insulin on tyrosine phosphorylation of pp185, and insulin receptor (IR) autophosphorylation. Serum-starved CHONeoB, CHONewIRa, and CHOK1018A cells were incubated with or without insulin. **A:** 100 μg of cell lysate was electrophoresed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). pp185 was detected by Western blots, using anti-phosphotyrosine PY20 antibody. **B:** WGA isolated IR receptors from CHONewIRa an CHOK1018A cells were exposed to insulin and ATP, resolved in a 7.5% SDS-PAGE, and detected by Western blot, using anti-phosphotyrosine PY20 antibody. A 15-min exposure is shown. **C:** WGA isolated IR receptors from CHONeoB cells exposed to insulin, resolved in a 7.5% SDS-PAGE and detected by Western blot, using anti-phosphotyrosine PY20 antibody. A different length of exposure of 16 h was necessary to see the signals on this blot. Results are representative of two similar experiments.

suggesting that this mutation has a dominant-negative effect on receptor phosphorylation. The phosphorylated insulin receptor becomes an active tyrosine kinase capable of phosphorylating substrates inside the cell. Two of these substrates, insulin receptor substrate 1 (IRS-1) and IRS-2, have a molecular size of about 180,000 and were initially identified as pp185. As shown in Figure 1C, insulin stimulation of pp185 tyrosine phosphorylation was higher in CHONewIRa cells and correlated with insulin-stimulated receptor phosphorylation. These data confirm that the cells used had low levels of endogenous insulin receptors (CHONeoB), high levels of normal insulin receptors (CHONewIRa), or insulin receptors devoid of tyrosine kinase activity (CHOK1018A).

### Effect of Insulin on Gene 33 mRNA Levels

CHO cells were incubated with 14 nM insulin for 1, 3, and 5 h. Maximal insulin induction of g33 mRNA levels was observed at 3 h for CHONeoB and CHONewIRa cells (Fig. 2A). Conversely, maximum effect was observed after 1 h of insulin exposure in CHOK1018A cells. A 6.95 ± 1.15-fold induction was evident in the CHONewIRa cells, followed by CHOK1018A (2.13 ± 0.42), and CHONeoB (1.62 ± 0.32).

We then examined the effect of increasing concentrations of insulin on g33 mRNA levels. CHONeoB and CHONewIRa cells were exposed for 3 h, and CHOK1018A for 1 h, based on the results of the time-course experiments (Fig. 2A). The g33 mRNA levels increased in a dose-dependent manner (Fig. 2B). However, the minimal response in CHONeoB and CHOK1018A cells prevented a mathematical analysis of the dose-response curve. In CHONewIRa, cells the maximal stimulation was 3.29 ± 0.08-fold above basal and half-maximal stimulation was observed at 0.06 ± 0.01 nM of insulin, consistent with insulin interaction with its own high-affinity receptor.

### Effect of Insulin Signaling Inhibitors on Stimulation of g33 mRNA Levels

The activation of IRS-1 and IRS-2 by the insulin receptor stimulates a number of intracellular signaling pathways, which have been involved in the mitogenic and metabolic action of the hormone. It is not known which of these pathways is required for insulin stimulation of g33 mRNA levels. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, at concentrations of ≤100 nM, which inhibits insulin stimulation of glucose transport in CHO cells (Table II) caused a small reduction of the mean values of $mRNA^{g33}$ expression (Fig. 3A) in CHONewIRa cells. It prevents the phosphorylation of pAKT, which is a downstream target of PI 3-K. Rapamycin, a p70 S6 kinase inhibitor, did not alter the relative amounts of $mRNA^{g33}$ when administered in conjunction with insulin. However, it successfully blocked the p70S6 kinase substrate p31 S6 protein phosphorylation (Fig. 3C). PD98059, an inhibitor of mitogen-activated kinase kinase (MAPKK), at concentration of 100 μM is reduced by 25% insulin (140 nM) stimulation of g33 mRNA levels in CHONewIRa cells (Fig. 3A). It inhibited the phosphorylation of the MAPK isoform p44 (Fig. 3C). Lower doses of wortmannin, rapamycin, and PD98059 were without effect in CHONewIRa and all other cell lines (CHONeoB and CHOK1018A) (not shown). $HNMPA(AM)_3$, an inhibitor of the insulin receptor [Saperstein et al., 1989], was the only compound that completely inhibited insulin-stimulated expression of $mRNA^{g33}$ (Fig. 3B). It also affected the phosphorylation of pAKT and pMAPK but not p31 S6 (Fig. 3C).

Half-maximal inhibition of insulin effect was observed with 40.2 μM $HNMPA(AM)_3$ in CHONewIRa cells. The possible cytotoxic effects of $HMNPA(AM)_3$ were evaluated using the Trypan blue exclusion test. CHO cells lines used were exposed to 117 μM of $HNMPA(AM)_3$ in the presence of 140 nM insulin, and compared with untreated cells and cells treated with 140 nM insulin. The percentage of viable cells for all cell lines induced with 140 nM insulin ranged from 86% to 88%. Cells treated with 117 μM $HNMPA(AM)_3$ in the presence of insulin, had viabilities within the range of 83% to 91%. When exposed to 117 μM $HNMPA(AM)_3$, viability was around 87%. These analyses indicate that the concentrations of insulin and the inhibitor used in our experiments were not toxic at the concentration tested. Each of the inhibitors tested, using exactly the same conditions, was able to inhibit their target proteins on insulin-stimulated CHO NewIRa cells (Fig. 3C).

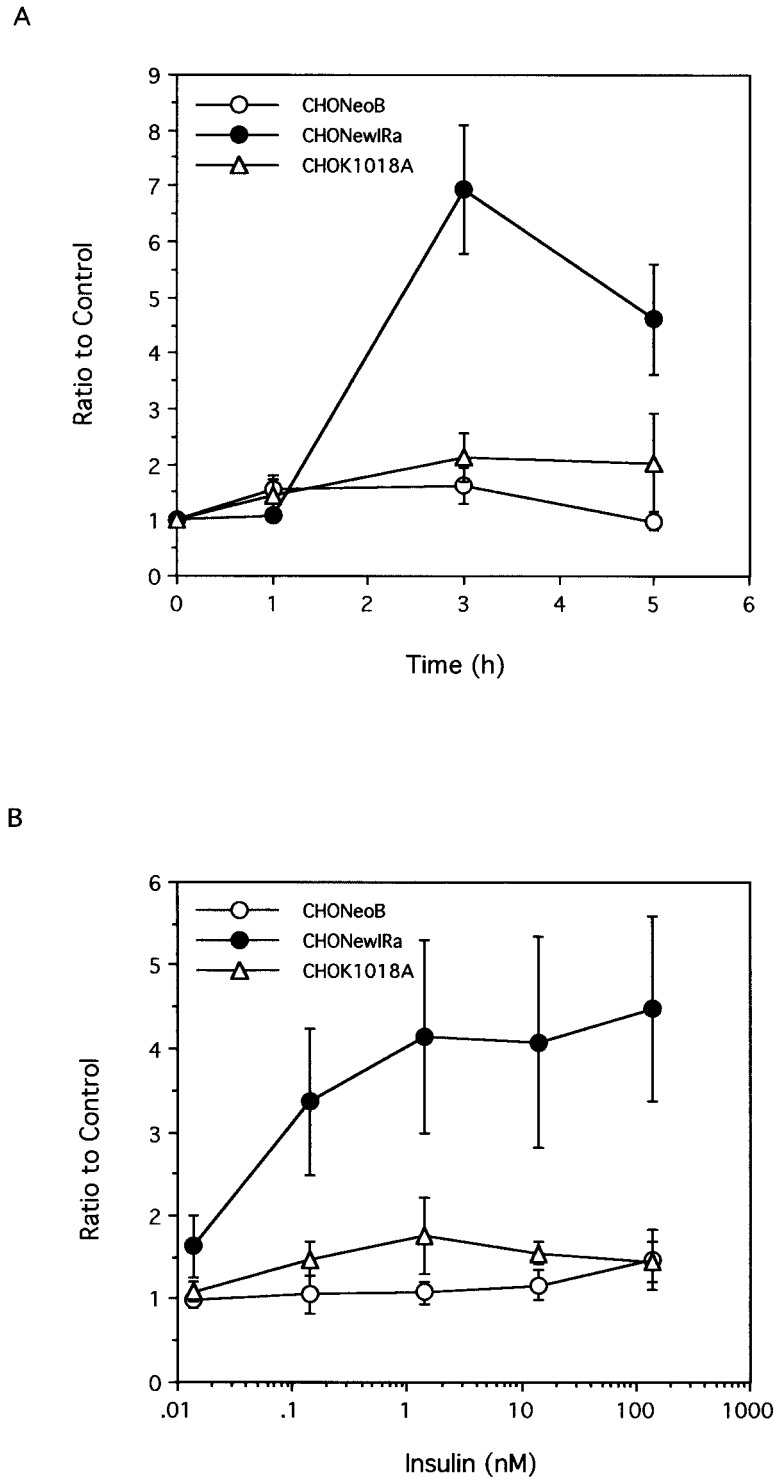


**Fig. 2.** Insulin time- and dose-dependent g33 mRNA expression in Chinese hamster ovary (CHO) cell clones. CHO cells were cultured in 1% fetal bovine serum (FBS) Ham's F-12 medium for 16–24 h before insulin treatments. Total RNA (30 μg) was run on denaturing agarose gels, blotted, and probed with $^{32}$P-labeled probes for g33 and α-tubulin and analyzed, using a PhosphorImager as described under Experimental Procedures. The g33 signals were standardized by dividing by the α-tubulin signal and expressed as a ratio to control samples. **A:** Insulin (14-nM) treatment of CHO cell lines for the indicated times. Averages ±SEM of three to five independent experiments are shown. **B:** In these dose-response experiments, CHONeoB and CHONewIRa cells were exposed to the indicated increasing amounts of insulin for 3 h. CHOK1018A cells were insulin-treated for 1 h. The values presented are means ±SEM of three to six independent experiments.

**TABLE II. Glucose Transport (OMG) of CHO NewIRa Cells Expressing Normal Insulin Receptors***

| Treatment | OMG transport nmol/ml/s | % Change |
|---|---|---|
| Basal | 3.00 ± 0.24 | 100 |
| Insulin, 200 nM | 9.15 ± 0.33 | +205 |
| Wortmannin, 100 nM | 2.24 ± 0.37 | −25 |
| Wortmannin + insulin | 2.45 ± 0.35 | −18 |

*Experiments were performed as previously described [Longo et al., 1992]. Cells were plated into 24-well plates and grown to confluence. Wortmannin was added 5 min before 1-h insulin stimulation (200 nM). 3-O-Methyl[$^3$H]-D-glucose (OMG) (1-mM) transport was then measured for 20 s. Points are averages of triplicates ±SD.

## DISCUSSION

Gene 33 is expressed in rat liver and rat hepatoma cell lines (H4IIE) and there is a rapid increase in gene transcription upon insulin stimulation [Lee et al., 1985; Messina et al., 1985]. However, the mechanism used by insulin to accomplish this action has not been defined. In this paper, we report the first attempt to study in detail how insulin increases the levels of the endogenous gene 33 mRNA in CHO cells stably-transfected with normal and mutant human insulin receptors. We decided to use CHO cells for two reasons: (1) their endogenous levels of insulin receptors are low compared with rat hepatoma cells and adipocytes (which average $2 \times 10^5$ IR/cell), (2) there are CHOhIR stable mutants which allow to determine the requirement for the kinase activity of the receptor.

The cells used in this research included CHO cells with only their endogenous insulin receptors (CHONeoB, 5,800 receptors/cell), CHONewIRa transfected with the normal human insulin receptor cDNA (93,200 receptors/cell), and CHOK1018A cells, expressing about 39,000 kinase-defective receptors in addition to their endogenous receptors (Table I). Phosphorylation experiments confirmed that CHOK1018A cells had kinase defective receptors, while the normal insulin-stimulated increase of receptor autophosphorylation and kinase activity toward endogenous substrates could be observed in CHONewIRa cells (Fig. 1). Insulin increased g33 mRNA levels in a time-dependent manner, with maximal expression after 1–3 h (Fig. 2A). At 5 h, the g33 mRNA levels declined, consistent with g33 as an immediate-response gene. The levels of g33 mRNA were increased only slightly in CHONeoB and in CHOK1018A cells, as compared to CHONewIRa cells where the induction was four- to sixfold higher (Fig. 2). The small increase in g33 mRNA levels in both CHONeoB and CHOK1018A cell lines were probably due to the presence of active endogenous CHO insulin receptors. It was previously shown [Messina et al., 1985] in H4 rat hepatoma cells, which have as much as 200,000 IR/cell, that a 2-h treatment with 5 nM insulin, increased the levels of gene 33 14-folds, eventually decreasing after 4 h. Our results suggest that high levels of insulin receptor are necessary for insulin stimulation (>2-fold) of g33 mRNA levels. The minimal effect observed in CHONeoB and CHOK1018A cells demonstrates that a functional insulin receptor is required for this effect. The CHO gene 33 responded to insulin in a dose-dependent manner, with half-maximal stimulation being observed at 0.06 ± 0.01 nM in CHONewIRa cells (Fig. 2B). In these cells, an effect (3.4-fold induction) was observed at concentrations as low as 0.14 nM of insulin. Messina et al. [1985] demonstrated that insulin concentrations as low as $10^{-11}$ M are capable of provoking a 2-fold increase in g33 mRNA in rat hepatoma cells, suggesting that the effects of the hormone are mediated through high-affinity insulin receptors, and not through interactions with other growth factor receptors. By contrast, Granner and coworkers [Chu et al., 1988] observed a modest increase at insulin concentrations of $10^{-12}$ to $10^{-10}$ M, using H4IIE cells, with the largest changes occurring at higher concentrations. Our results provide the final demonstration that the wild-type insulin receptor, present in high levels in the cell, is necessary for the insulin response of g33.

Our levels of induction, time-course, and dose-response curve compared well with the response of hexokinase II and c-*fos* to insulin. Induction of hexokinase II mRNA by insulin (10 nM) occurs within a short time (2 h) and is maximal at 4 h in adipose and muscle cells [Printz et al., 1993]. Similarly, insulin (5 nM) elevates mRNA$^{c\text{-}fos}$ levels to 6.5-fold after 15 min of exposure using H4IIE cells [Messina, 1990] and a low insulin concentration (0.5 nM) is sufficient to exert 3.5-fold induction. Similar data were obtained in Rat-1 fibroblasts cells overexpressing normal [Jhun et al., 1995]

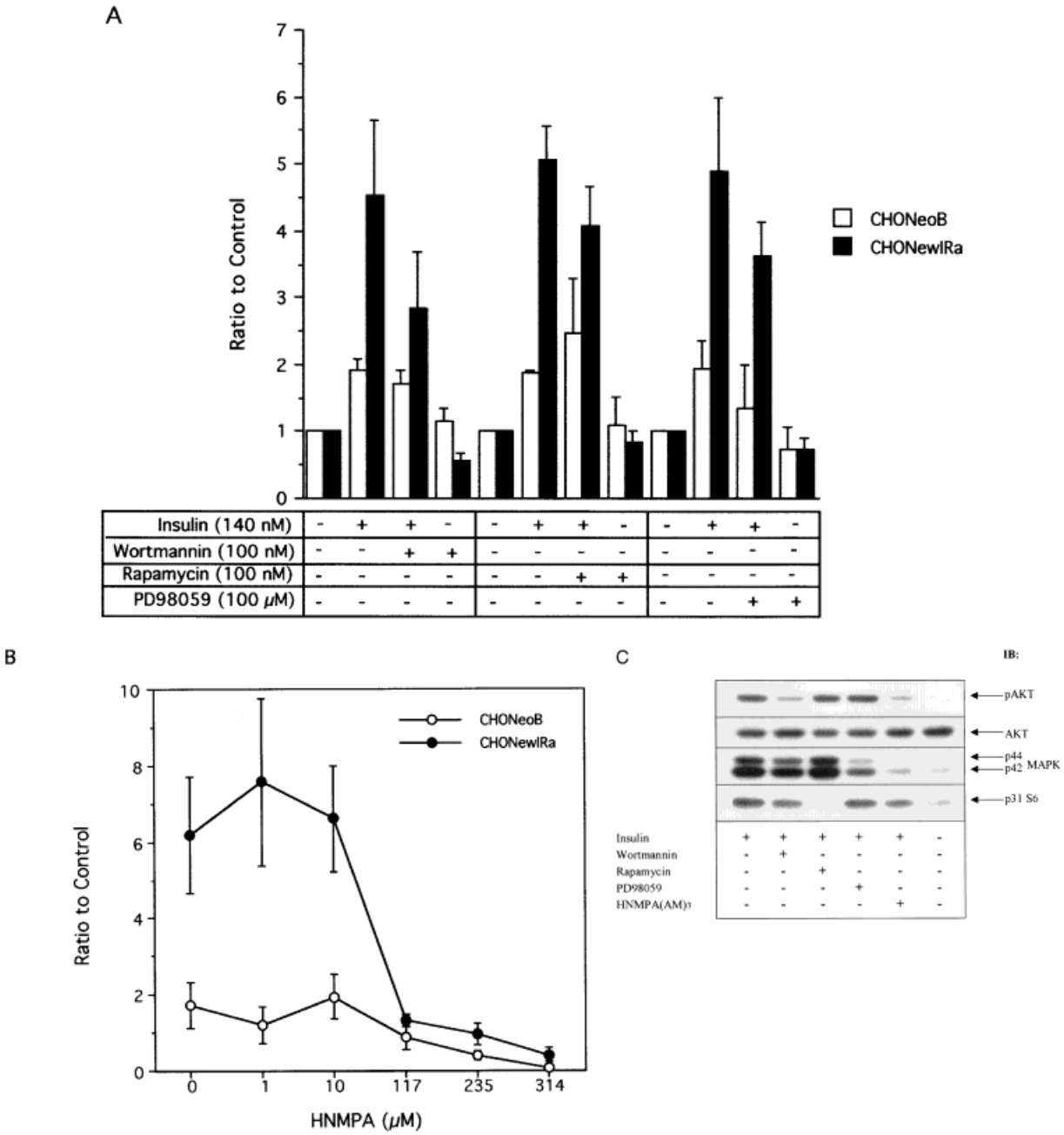


**Fig. 3.** Effects of wortmannin, rapamycin, and PD98059 on insulin-induced g33 mRNA expression. CHONeoB and CHONewIRa cells were cultured in 1% fetal bovine serum (FBS) Ham's F-12 medium 16–24 h before the experiments. Total RNA (30 μg) was run on denaturing agarose gels, transferred, and reacted with $^{32}$P-labeled probes for g33 and α-tubulin and analyzed, using a PhosphorImager as described under Experimental Procedures. The g33 signals were normalized by dividing by the α-tubulin signal, and expressed as a ratio to control samples. **A:** Chinese hamster ovary (CHO) cells were incubated with the indicated concentration of the inhibitors for 15–30 min and then incubated in the absence or presence of 140 nM insulin for 3 h in CHONeoB and CHONewIRa cells and for 1 h in CHOK1018A cells. **B:** CHO cells were incubated with the indicated concentrations of HNMPA$(AM)_3$ for 1 h and then incubated in the absence or presence of 140 nM insulin for 3 h. Results shown represent the mean ±SEM of three independent experiments. **C:** CHO NewIRa cells were incubated with or without wortmannin (100 nM), rapamycin (100 nM), PD98059 (100 μM), and HNMPA ($AM_3$) (117 μM) for 30 min, with or without insulin (100 nM) for 1 h. A total of 30 μg of total protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (SDS-PAGE) and immunoblotted sequentially with pAKT, AKT, pMAPK, and p31 S6 antibodies.

but not mutant [Harada et al., 1995] insulin receptors.

Selective inhibitors of insulin signaling were used to determine the pathway required for insulin stimulation of $mRNA^{g33}$ levels. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, caused a reduction of the mean values of g33 mRNA expression in CHONewIRa from 4.4- to 2.8-fold (Fig. 3A), which was not statistically significant. This relatively low effect suggests that phosphatidylinositol 3-kinase does not play the only role in gene 33 regulation by insulin in CHO cells. Alternatively, higher concentrations of wortmannin (>500 nM) may be required to block the enzyme, as the inhibitor has been shown to lose effectiveness after approximately 2 h of incubation at physiological pH [Nakanishi et al., 1992; Sutherland et al., 1995; Kimura et al., 1994; Woscholski et al., 1994]. Although we did not add inhibitor through the incubation again, the use of a similar experimental scheme and a lower concentration of wortmannin by others [Yang and Dickson 1995a, b] could prevent insulin stimulation of g33 mRNA levels in rat hepatoma cells. The apparent lack of effectiveness of wortmannin in our cells should be viewed in the light of possible differences in cell specific sensitivity due to variable levels of insulin receptor isoform expression and/or instability of the inhibitor. Alternatively, $mRNA^{g33}$ levels may not be regulated through the well-known PI 3-K pathway. However, we showed by Western blot analysis that the phosphorylation of AKT, one of the downstream targets of PI 3-K, was abolished using the same conditions (100 nM). Other investigators have been able to demonstrate the involvement of AKT in the transcriptional regulation by insulin in the fatty acid synthase, Glut-1, and glucose-6-phosphatase genes [Wang and Sul, 1998; Barthel et al., 1999; Dickens et al., 1998].

Rapamycin has been used extensively to evaluate the effects of insulin action on several genes, such as PEPCK [Yamauchi et al., 1993; Sutherland et al., 1995], and glycogen synthase [Sakaue et al., 1995; Azpiazu et al., 1996; Cross et al., 1994; Shepherd et al., 1995], as its inhibitory effects appear to be selective for the p70S6 kinase pathway without affecting PI 3-kinase, MAP kinase (Fig. 3C), or p90rsk enzyme activity [Chung et al., 1992; Price et al., 1992]. Rapamycin at 100 nM inhibited p31 S6 phosphorylation (Fig. 3C). Sakaue et al. [1995] and Kim and Kahn [1997] showed that at 22 nM inhibits the p70 S6 kinase in CHO-IR cells. We did not observe any inhibitory effect of rapamycin on basal or insulin-induced $mRNA^{g33}$ in CHONewIRa cells (Fig. 3A). These findings confirm a previous report indicating the lack of the effect of rapamycin on insulin induction of gene 33 and phosphoenolpyruvate carboxykinase mRNA in rat hepatoma cells [Yang and Dickson, 1995a]. Recent reports suggest that activation of this Ras/MAP kinase pathway is critical for the effects of insulin on mitogenesis, c-*fos* [Lazar et al., 1995] and *egr-1* gene expression (Harada et al., 1996). However, this pathway is not required for insulin or phorbol esters regulated gene expression of phosphoenolpyruvate carboxykinase [Gabbay et al., 1996] and hexokinase II genes [Osawa et al., 1996].

PD98059 is a selective reversible inhibitor of MAPKK [Dudley et al., 1995], which does not inhibit significantly the activity of MAPK itself and permits examination of the role of p42/p44 MAP kinase in the insulin-regulated activation of g33 mRNA (Fig. 3C). Insulin-stimulated CHONewIRa, CHONeoB, and CHOK1018A cells were exposed to increasing concentrations of PD98059. PD98059 (100 μM) reduced the effect of insulin on gene 33 expression from five- to fourfold in CHONewIRa cells (Fig. 3A). This effect was not statistically significant, suggesting that activation of p42/p44 MAP kinase does not have a major role in insulin activation of g33.

$HNMPA(AM)_3$, an inhibitor of the insulin receptor tyrosine kinase was designed and synthesized [Saperstein et al., 1989]. This compound inhibited insulin stimulation of gene 33 expression in all cells lines tested (Fig. 3B). Half-maximal inhibition was observed at 40.2 μM in CHO NewIRa cells, which is within those observed for half-maximal inhibition of the receptor tyrosine kinase (100 μM) and insulin stimulation of glucose oxidation in adipocytes (10 μM) [Saperstein et al., 1989]. This inhibition was not attributable to a generalized toxic effect of the vehicle (DMSO) or by $HNMPA(AM)_3$, as Trypan blue exclusion assays were comparable in both treated and untreated cells. These results agree with previous observations [Saperstein et al., 1989], indicating that concentrations as high as 100 μM do not affect [$^3$H]thymidine incorporation in CHOhIR cells. The precise step at which $HNMPA(AM)_3$ exerts its action has not been

yet determined. However, in our system it inhibited IR and pp185 phosphorylation in total cell lysates (data not shown). Nevertheless, $HNMPA(AM)_3$ does not affect insulin binding but reduces by 50% insulin receptor autophosphorylation [Baltenspenger et al., 1992] and tyrosine kinase activity toward exogenous substrates like angiotesin II [Saperstein et al., 1989].

The regulation of gene 33 is another example of a highly insulin-regulated gene that may use various cell-specific pathways. Yet further studies are needed to elucidate the complicated insulin signal transduction network. Our data suggest that pAKT may be involved in g33 mRNA insulin-induced activation in CHONewIRa cells.

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