Insulin Stimulates cAMP-response Element Binding Protein Activity in HepG2 and 3T3-L1 Cell Lines*

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Insulin binding to its cell surface receptor results in alterations in the expression of many genes for cellular growth, differentiation, and proliferation. Specific insulin responsive elements have been identified in the promoters of several genes (1–4). Peroxisome proliferator-activated receptors and other steroid hormone receptors have been implicated in regulating gene transcription through these insulin responsive elements (2, 4, 5). In other genes, insulin responsive sites have been mapped to regions containing cAMP responsive elements (CREs) and serum responsive elements (6–9). Consistent with these findings, many insulin-regulated genes are also regulated by extracellular stimuli that modulate intracellular cAMP levels (2, 10–17) (Table I). Previously, we demonstrated that phosphorylation of CREB was stimulated by insulin in primary rat adipocytes and HIRc (18, 19). This observation posed an important question regarding the impact of insulin-mediated CREB phosphorylation on CREB transactivation and the post-receptor pathways activated by insulin responsible for this effect.

Cyclic AMP regulates the transcription of target genes primarily through the phosphorylation of the cAMP-response element binding protein (CREB) by protein kinase A (PKA) on serine 133 (of CREB-347 or serine 119 of CREB-327) of the CREB molecule (20–23). We demonstrated an analogous response to insulin and identified that this increase in phosphorylation was at least in part due to a decrease in nuclear protein phosphatase-2A activity. These experiments were the first to show a transient increase in CREB phosphorylation and regulation of a nuclear, serine/threonine-specific protein phosphatase in response to activation of a tyrosine kinase growth factor receptor. Simultaneously, Ginty et al. (24) described a similar increase in CREB phosphorylation in response to nerve growth factor (NGF), which binds to another tyrosine kinase growth factor receptor, in PC12 cells. It was stated in this paper that CREB phosphorylation alone by NGF was not sufficient to activate a Gal-4 CREB reporter. Since the time of these initial observations, an explosion of new data on growth factor, Ca2+, and cytokine regulation of CREB has emerged, and a number of post-receptor signaling pathways including ERK 1/2 pathway, pp70 S6 kinase pathway, p38 pathway, and PI-3 kinase pathway have been implicated in CREB regulation.

In this paper, we demonstrate that insulin stimulates the phosphorylation of CREB at serine 133 and thereby enhances CREB transcriptional activity in the HepG2, human hepatoma cells, and in mouse 3T3-L1 fibroblasts and adipocytes. CREB phosphorylation and activation increased rapidly following the addition of physiological concentrations of insulin, but no change in CREB DNA binding activity was observed. Pharma-
The abbreviations used are: Stim, stimulatory; Inhib, inhibitory.

**TABLE I**

| Gene                                      | cAMP effect | Insulin effect | Ref. |
|-------------------------------------------|-------------|----------------|------|
| Phosphoenolpyruvate carboxykinase (Cytosolic) | Stim*        | Inhib*         | 7, 8 |
| Fructose-1,6-bisphosphatase                | Stim         | Inhib          | 12   |
| Aspartate aminotransferase (Cytosolic)     | Stim         | Inhib          | 10   |
| Insulin-like growth factor binding protein 1 | Stim         | Inhib          | 17   |
| L type pyruvate kinase                    | Inhib        | Stim           | 8    |
| Fatty acid synthase                       | Inhib        | Stim           | 2    |
| Glutamate-3-phosphate                     | Inhib        | Stim           | 15   |
| Acetyltransferase (mitochondrial)         |              |                |      |
| Glucokinase                               | Inhib        | Stim           | 14   |
| 6-Phosphofructo-1-kinase                  | Inhib        | Stim           | 13   |
| Fructose-2,6-bisphosphatase/6-             | Inhib        | Stim           | 11   |
| phosphofructo-2-kinase                    |              |                |      |
| AcetylphosphoCoA carboxylase c-fos         | Stim         | Stim           | 16   |
|                                       | Stim         | Stim           | 9    |

* The abbreviations used are: Stim, stimulatory; Inhib, inhibitory.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media and supplies were from Life Technologies, Inc. (Beverly, MA), Gemini Bioproducts (Gaithersburg, MD), and Specialty Media, Inc. (Lavallette, NJ). 3T3-L1 fibroblasts were provided by Dr. Ted Ciraldi (La Jolla, CA). Luciferase assay reagents were obtained from Analytical Luminescence Laboratory (San Diego, CA), and chloramphenicol acetyltransferase enzyme-linked immunosorbent assay kits were from Boehringer Mannheim. A plasmid containing an enhancerless thymidine kinase (TK) promoter linked to four copies of the Gal4 regulatory sequence driving expression of a luciferase reporter gene (pGal4TK-LUC) was provided by Dr. James Hoeffer (Intragen, San Diego, CA). Expression vectors for Gal4-CREB proteins with serine to alanine substitutions at amino acids 117, 129, and 133 in the CREB transactivation region linked to the Gal4 DNA binding domain (designated Gal4-CREB-341 S117A, S129A, and S133A, respectively) were generated as described elsewhere (25). An expression vector (pRSV-KCREB) for the dominant negative CREB inhibitor protein, KCREB, was provided by Dr. Richard Goodman (Oregon Health Sciences University, Portland, OR). Plasmids for the expression of constitutively active Ras (pSvRas), dominant negative Raf (pRSV4B Raf) were supplied by Dr. Ulf Rapp (Strathlkenbein, Germany). 3P]orthophosphate was purchased from ICN (Irvine, CA). Okadaki acid was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). CREB- and P-CREB-specific antibodies were purchased from New England Biolabs (Beverly, MA). All other reagents were of molecular biology grade or better and were purchased from Sigma. Rapamycin and wortmannin were obtained from BioMol (Plymouth Meeting, PA), and PD98059 was a gift from Parke-Davis.

**Cell Lines and Transfection Procedures**—HepG2, human hepatoma cells, were obtained from the American Type Culture Collection (Rockville, MD) and grown in low glucose Dulbecco's medium (DMEM/F12 containing 5% fetal calf serum (FCS), and 5% calf serum. 3T3-L1 fibroblasts were a gift of Ted Ciraldi (University of California, San Diego). 3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and grown in low glucose Dulbecco's medium plus 10% FCS, 50 μg/ml gentamicin, 1 mM l-glutamine, and 450 ng/ml fungizone. 3T3-L1 fibroblasts were differentiated into adipocytes after reaching confluency by the addition of differentiation medium (high glucose DMEM containing 10% FCS, 50 μg/ml gentamicin, 1 mM l-glutamine, 500 μM isobutylmethylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin). After 2 days, the 3T3-L1 cells were transferred to adipocyte growth medium (high glucose DMEM plus 10% FCS, 50 μg/ml gentamicin, 1 mM l-glutamine, and 1 μg/ml insulin) and refed every 2 days (26). Differences between mature adipocytes were confirmed by Oil Red staining of lipid vesicles.

Plates of HepG2 and 3T3-L1 fibroblasts and adipocytes were grown to 70–80% confluency and transfected with the indicated plasmids by calcium phosphate-DNA coprecipitation as described by Wadzinski et al. (27) or using LipofectAMINE according to the manufacturer's directions. Cells were treated with insulin and/or other reagents at the concentrations and times specified in the figure legends. Luciferase assays were performed on a Monolight 1010 luminometer using the Enhanced Luciferase Assay kit (Analytical Luminescence Laboratory, San Diego, CA) according to the supplier's directions. Transfection efficiencies were normalized by cotransfecting the cells with a plasmid containing a chimeric SV40 promoter/galactosidase gene, and β-galactosidase levels were measured as described previously (27). All experiments were repeated at least three times, and consistent results were obtained in all cases.

**Immunoprecipitation and Tryptic Phosphopeptide Mapping of 32P-CREB**—HepG2 and 3T3-L1 fibroblasts and adipocytes were grown to approximately 80% confluency as described above. Plates of cells (2.5 × 10⁶ cells) were washed with phosphate-buffered saline, and the medium was replaced with 5 ml of phosphate-free DMEM containing 1% bovine serum albumin and 1 mM of [32P]orthophosphate. The plates were incubated 4–16 h at 37°C and then treated with insulin and/or other agents for the indicated times.

At each time point, the medium was removed from the cells, which were washed once with cold phosphate-buffered saline. The cell pellets were lysed in 1 ml of 20 mM Hepes, pH 7.9, containing 1% sodium dodecyl sulfate (SDS), and 0.1% 2-mercaptoethanol in a boiling water bath for 2 min. The lysates were diluted with 9 ml of 20 mM Hepes, pH 7.9, containing 1% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 mM benzamidine, 1 μg/ml β-mercaptoethanol, 2 mM sodium vanadate, 50 mM sodium fluoride, 50 μM phenylarsine oxide, and 100 μM okadaic acid. After normalizing the supernatants for protein concentration, CREB was recovered from the supernatants by immunoprecipitation with CREB-specific antibodies covalently attached to protein-A-Sepharose beads with dimethyl pimelimidate (27).

Immunoprecipitated material was resolved on 10% polyacrylamide-SDS gels. The identity of the CREB band and the relative amounts of CREB recovered at each time point were determined by Western blotting using a phopho-CREB-specific antibody and comparing the positions of the bands with those of a standard of purified CREB.

**Western Blot Analysis of CREB and P-CREB**—Lysates from HepG2 cells and 3T3-L1 fibroblasts and adipocytes treated with insulin and/or other reagents were prepared as described above at the times indicated in the figure legends. After correcting for protein concentrations, the lysates were resolved on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. The nitrocellulose blots were blocked with phosphate-buffered saline containing 5% dry milk and 0.1% Tween 20 and then treated with antibodies that recognize phosphorylated CREB (P-CREB) alone or that recognize both unphosphorylated and phosphorylated forms of CREB. The blots were washed and subsequently treated with goat anti-rabbit IgG conjugated to alkaline phosphatase. After the blots were washed, specific immune complexes were visualized with bromochloroindolyl phosphate and nitro blue tetrazolium.

In some experiments, proteins were transferred to PVDF membranes that were then blocked in 20 mM Tris-HCl, pH 7.9, 8.5% NaCl (TBS) containing 5%Blocking Grade Non-Fat Dry Milk and 0.1% Tween 20 for 1 h (Blocking Buffer). The blots were then incubated with the indicated primary antibodies in TBS containing 5% bovine serum albumin and 0.1% Tween 20. Membranes were then rinsed three times in Blocking Buffer and then twice in 10 mM Tris-HCl, pH 9.5, 10 mM NaCl, 1 mM MgCl₂. Blots were then incubated with 1:500 dilution of CDP-Star reagent prepared in 1× Assay Buffer (both reagents from New England Biolabs, Beverly, MA) for 5 min and then exposed to film.

**Electrophoretic Mobility Shift Assay**—The binding affinities of unphosphorylated CREB and phosphorylated CREB in response to insulin were calculated from electrophoretic mobility shift assay data obtained from phospho-CREB mobility shift assay performed with concentrations of 32P-labeled CREB probe from 0.1 to 20 ng per 20-μl reaction. The binding of CREB to 32P-labeled probe was saturable at high probe concentrations and was found to be reversible since the addition of unlabeled CRE probe to electrophoretic mobility shift assay reactions prior to electrophoresis decreased CREB DNA binding as compared with reactions with no added CRE probe.
with no unlabeled probe. Nuclear extract preparations, electrophoretic mobility shift assays, and Scatchard analysis of equilibrium binding data were performed as described previously (29, 30).

RESULTS

Insulin Stimulates Phosphorylation of CREB at Serine 133—A number of laboratories have reported that the activation of certain tyrosine kinase/growth factor receptors, such as NGF receptor, epidermal growth factor receptor (24), and fibroblast growth factor receptor (4), increases the phosphorylation and transcriptional activity of the nuclear protein, CREB. In one of the first reports of this phenomenon, we demonstrated that insulin produced a transient increase in CREB phosphorylation in primary rat adipocytes (18). Our initial experiments also showed that insulin transiently decreased nuclear protein phosphatase 2A activity that could partly account for increased CREB phosphorylation (18, 19). However, the effect of insulin on CREB transcriptional activity was not addressed nor was the site(s) at which CREB was phosphorylated in response to insulin identified.

Insulin Stimulates CREB Phosphorylation at Serine 133 in a Time- and Dose-dependent Manner—To assess further the impact of insulin on CREB phosphorylation, we used two cell lines representative of normal insulin-responsive tissues: HepG2 hepatoma cells derived from human liver and 3T3-L1 fibroblasts and adipocytes as representatives of adipose tissue. As shown in Fig. 1A, both insulin and Bt2cAMP rapidly increased the level of 

Insulin Enhances CREB Transcriptional Activity

Phosphorylation of CREB at serine 133 increases the transcriptionsal transactivation activity of the protein. To assess the ability of insulin to enhance CREB transcriptional activity, we measured the ability of insulin to regulate a luciferase reporter gene linked to the herpes simplex virus-thymidine kinase promoter containing four copies of the Gal4 response element (pGal4TK-LUC) in HepG2 cells and 3T3-L1 fibroblasts and adipocytes. The cells were cotransfected with an expression vector from which a chimeric protein containing the Gal4 DNA binding domain linked to the CREB transactivation region was expressed (pRSV-Gal4). Since mammalian cells lack transcription factors that bind the Gal4 consensus sequence, this system directly measures the effect of insulin on the CREB transactivation region of the chimeric protein. Control cells did not receive the Gal4-CREB expression plasmid. Transcription from the Gal4-responsive promoter was unaffected by insulin treatment in HepG2 cells in the absence of Gal4-CREB protein (Fig. 2A). However, when cotransfected with the Gal4-CREB-341 expression vector, insulin stimulated transcription from the Gal4 responsive promoter by 4.5-fold ± 0.27. Likewise, insulin stimulated Gal4-CREB responsive transcription 8.5-fold in 3T3-L1 fibroblasts and 4.45-fold in adipocytes.

The effect of insulin on endogenous CREB protein in HepG2 cells was assessed by measuring transcription from an enhancerless SV40 promoter (pSV-luc) linked to three copies of a CRE consensus sequence (pCRESVluc) and a CREB-responsive/CRE-containing region of the somatostatin promoter (−71 to +53) (pΔ71Luc). Transcription from both promoters was stimulated approximately 4–5-fold following treatment with 10 nM insulin for 4 h (Fig. 2B). However, no increase in transcription was noted from an enhancerless SV40 promoter lacking any CRE sequences. Likewise, no stimulation of transcription was noted when any of these reporter plasmids were cotransfected with an expression vector for the dominant negative CREB inhibitor protein, KCREB (5).

The ability of insulin to stimulate the transcriptional activity of the chimeric Gal4-CREB protein and the inhibition of insulin-stimulated transcription from CRE-containing promoters by the CREB-specific inhibitor, KCREB, clearly demonstrate that insulin enhances CREB transcriptional activity in cell lines representative of typical insulin-responsive tissues. To apply these observations to physiologically relevant genes in adipocytes, we examined three promoters as follows: the full-length PEPCK promoter, the fatty-acid synthase promoter (pPASluc), and the fatty acid binding protein promoter (pFABPluc) (courtesy of Steve Clarke, Austin, TX) fused to a luciferase reporter construct. Insulin led to a 4–5-fold induction of luciferase activity from the PEPCK and FAS promoter with a less profound, but statistically significant, increase in the FABP promoter activity (Fig. 2B). It is important to note that whereas PEPCK gene transcription is down-regulated by insulin in hepatocytes it is stimulated in adipocytes. We did not examine transcription from the PEPCK promoter in HepG2
The cells were then treated with either 0.5 mM Bt2cAMP or 10 nM insulin for the times indicated above the figure. For cells grown in normal medium, the lysates were prepared at each time point, and equal amounts of lysate protein were separated on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. Separate blots were probed with antibodies specific for CREB phosphorylated at serine 133 (P-CREB Ab) or total CREB (CREB Ab). The positions of the P-CREB or CREB bands were identified from autoradiographs. A, HepG2 cells or 3T3-L1 fibroblasts or adipocytes. B, HepG2 cells or 3T3-L1 fibroblasts or adipocytes were transfected with either pGal4TK-Luc alone or cotransfected with p6Gal4TK-Luc and pRSV-Gal4-CREB as indicated. The following day, untreated, control cells (open bars) or cells treated with 10 nM insulin (HepG2) or 100 nM insulin (3T3-L1) for 4 h were lysed, and transcription levels (luciferase activity) were measured in the lysates. The figure shows data averaged from three to nine experiments. Levels of transcription are shown relative to levels measured in untreated cells cotransfected with the pRSV-Gal4-CREB plasmid. B, HepG2 cells were transfected with the plasmids pSV-Luc, pCRE-SV-Luc, or pP71-Luc, and 3T3-L1 fibroblasts were transfected with the plasmids pPPEPluc, pFAS-Luc, or pPABP-Luc as shown. As indicated, some cells were cotransfected with the plasmid, pRSV-KCREB. Each of these plasmids is described under “Experimental Procedures” and “Results.” The following day, untreated control cells (open bars) or cells treated with insulin (cross-hatched bars) as described above were lysed. Luciferase activity in the lysates was measured as an index of transcriptional activity, and the data shown were averaged from three to nine experiments. Levels of transcription are shown relative to levels in untreated cells not transfected with pRSV-KCREB.

The cells. The CREB protein was eluted and digested with trypsin. Peptides were separated on cellulose thin layer plates by electrophoresis at pH 1.9 from left to right (anode on the right), followed by chromatography from bottom to top. The figure shows autoradiograms of the thin layer plates. The origin where the peptides were spotted is indicated by the dark spots. The maps labeled Insulin + Bt2cAMP and Insulin + PKA Peptide were generated by mixing tryptic phosphopeptides of CREB from insulin-treated cells with either phosphopeptides of CREB from Bt2cAMP-treated cells or with a 32P-labeled synthetic peptide (RPSYR) comprising the CREB PKA recognition sequence, respectively. The “PKA site” or serine 133-containing peptides are indicated as described above. The CREB protein was eluted and digested with trypsin. Peptides were separated on cellulose thin layer plates by electrophoresis from left to right (anode on the right), followed by chromatography from bottom to top. The figure shows autoradiograms of the thin layer plates. The origin where the peptides were spotted is indicated by the dark spots. The maps labeled Insulin + Bt2cAMP and Insulin + PKA Peptide were generated by mixing tryptic phosphopeptides of CREB from insulin-treated cells with either phosphopeptides of CREB from Bt2cAMP-treated cells or with a 32P-labeled synthetic peptide (RPSYR) comprising the CREB PKA recognition sequence, respectively. The “PKA site” or serine 133-containing peptides are indicated as described above.

Fig. 2. Insulin stimulates CREB transcriptional activity in HepG2 cells and 3T3-L1 fibroblasts and adipocytes. A, HepG2 cells or 3T3-L1 fibroblasts or adipocytes were transfected with either pGal4TK-Luc alone or cotransfected with pGal4TK-Luc and pRSV-Gal4-CREB as indicated. The following day, untreated, control cells (open bars) or cells treated with 10 nM insulin (HepG2) or 100 nM insulin (3T3-L1) for 4 h were lysed, and transcription levels (luciferase activity) were measured in the lysates. The figure shows data averaged from three to nine experiments. Levels of transcription are shown relative to levels measured in untreated cells cotransfected with the pRSV-Gal4-CREB plasmid. B, HepG2 cells were transfected with the plasmids pSV-Luc, pCRE-SV-Luc, or pP71-Luc, and 3T3-L1 fibroblasts were transfected with the plasmids pPPEPluc, pFAS-Luc, or pPABP-Luc as shown. As indicated, some cells were cotransfected with the plasmid, pRSV-KCREB. Each of these plasmids is described under “Experimental Procedures” and “Results.” The following day, untreated control cells (open bars) or cells treated with insulin (cross-hatched bars) as described above were lysed. Luciferase activity in the lysates was measured as an index of transcriptional activity, and the data shown were averaged from three to nine experiments. Levels of transcription are shown relative to levels in untreated cells not transfected with pRSV-KCREB.
Fig. 3. Characterization of insulin-stimulated CREB transcriptional activity in HepG2 cells. A, HepG2 cells were cotransfected with pGal4TK-LUC and pHSV-Gal4-CREB-341. The following day the cells were treated with 10 nM insulin for the times indicated. Cells were lysed at each time point, and transcription levels (luciferase activity) were measured. Fold increases in transcription are relative to the level measured in untreated cells (no insulin). B, HepG2 cells were transfected with the plasmids described above and treated with the following day with the indicated concentrations of insulin for 4 h. Transcription (luciferase production) was measured in cell lysates, and the fold increases in transcription are relative to the level measured in untreated cells (not shown). C, Scatchard analysis of nuclear protein binding to a CRE probe was performed with nuclear extracts from untreated HepG2 cells (○) or from cells treated with 10 nM insulin for 5 min (□) or 2 h (▲). Approximately 5 μg of protein from each of the nuclear extracts was incubated in binding reactions containing [32P]labeled CRE probe ranging from 0.01 to 20 ng. The reactions were separated on 6% non-denaturing polyacrylamide gels that were subsequently exposed to film. Levels of bound and free probe in each lane were determined by densitometry of the resulting autoradiograph. The data represent the average of three experiments.

Fig. 4. CREB serine 133 is required for insulin-stimulated CREB transcriptional activity. 3T3-L1 fibroblasts were transfected with pGal4TK-LUC along with expression vectors for each of the wild type (WT) and mutant Gal4-CREB proteins indicated below the figure. The various Gal4-CREB plasmids and proteins are described under "Experimental Procedures" and "Results." Transfected HepG2 cells were cultured overnight in complete medium, whereas the 3T3-L1 cells were cultured in serum-free medium. The following day the cells were treated with either 10 nM (HepG2) or 100 nM (3T3-L1) insulin (cross-hatched bars). Untreated control cells and insulin-treated cells were lysed and luciferase activity measured as an index of transcriptional activity. Transcription levels are shown relative to levels measured in untreated HepG2 cells with no Gal4-CREB or untreated 3T3-L1 cells expressing wild type (WT) Gal4-CREB. The results were averaged for three to nine separate experiments. All constructs demonstrate statistically significant stimulation with insulin p < 0.05 except the pGal4CREB S133A and the Gal4CREB S82.

能力于胰岛素刺激的CREB转录活性。图3展示了结果。HepG2细胞单独与相似结果被观察到在3T3-L1细胞（未显示）。当HepG2细胞用10 nM胰岛素，Gal4-CREB介导的转录线性地从0到4 h处理（图3A）。转录水平 remained elevated for at least 8 h and then began to decline to basal levels (not shown). The time course for transcription did not match the time course for CREB phosphorylation shown in Fig. 1. However, similar differences have been reported for the time courses of transcription (luciferase expression) and CREB phosphorylation in response to cAMP analogs and appear to be due to the lag in overall luciferase expression as compared with the more rapid response of transcription alone. Gal4-CREB responsive transcription was also dependent on the concentration of insulin used to treat the cells (Fig. 3B). Transcription levels increased with insulin concentrations from 10^{-11} to 10^{-9} M. Concentrations of insulin higher than 10^{-9} M did not produce higher levels of transcription. Thus, the ability of insulin to enhance CREB transcription occurs rapidly and at physiological concentrations of insulin.

Scatchard analysis of CREB DNA binding activity determined by gel retardation assay indicated that insulin had no effect on the ability of CREB to bind CRE sequences (Fig. 3C).

Insulin Stimulates CREB Transcriptional Activity through the Phosphorylation of Serine 133—Our initial results demonstrated that insulin stimulated the phosphorylation of serine 133 of CREB. To confirm further the importance of serine 133 phosphorylation for CREB transcriptional activity, we next analyzed the ability of insulin to stimulate transcription from the Gal4-responsive promoter in the presence of wild type Gal4-CREB or Gal4-CREB proteins containing various mutations in the CREB transactivation domain (25). The wild type and mutant Gal4-CREB proteins were expressed at equivalent levels in these experiments as determined by Western blot analysis of cell lysates with Gal4-specific antibodies (data not shown). The data in Fig. 4 show that mutation of serine 133 to alanine completely blocks the ability of insulin to stimulate Gal4-CREB transcriptional activity in both HepG2 cells and 3T3-L1 fibroblasts. However, insulin was able to stimulate the transcriptional activity of other Gal4-CREB proteins having adjacent serines mutated to alamines (Gal4-CREBs S117A and S129A). Levels of basal transcription with these proteins was typically lower than observed with the wild type Gal4-CREB protein.

Effect of Pharmacologic Inhibitors on CREB Phosphorylation and Activity—Recent data indicate that multiple signal transduction pathways are capable of regulating CREB serine 133 phosphorylation (24, 33–39). Insulin-regulated pathways that have been implicated for serine 133 phosphorylation by other growth factors, cytokines, and cytosolic calcium include MAP
30 min or 30 nM PD98059 for 60 min were subsequently incubated with (relative to levels measured in cells not treated with insulin or any index of transcriptional activity. Levels of transcription are shown bars described above were subsequently incubated without (control) or cells pretreated with rapamycin, wortmannin, or PD98059 as overnight in serum-free medium. Untreated 3T3-L1 fibroblasts (cotransfected with pGal4TK-LUC and pRSV-Gal4-CREB-341) and piR-SV-Gal4-CREB-341. As indicated below the figure, the cells were also cotransfected with expression vectors for constitutively active Ras (+Ras) or Raf (+Raf), or dominant negative Ras (−Ras) or Raf (−Raf). Control cells (Ctrl) did not receive additional plasmids. After overnight incubation in serum-free medium, untreated cells (open bars) or cells treated with 100 nM insulin (cross-hatched bars) were lysed, and luciferase activity was measured in the lysates as an index of transcriptional activity. The data are averages from three experiments and are shown relative to levels measured in untreated, control cells.

To correlate these observations with transcriptional activity, we measured the effect of these agents on the Gal4-CREB-responsive transcription system. In these experiments, PD98059 completely inhibited insulin-stimulated transcription from the Gal4-CREB-responsive promoter (Fig. 5A). Rapamycin and wortmannin did not inhibit insulin-stimulated/Gal4-CREB-mediated transcription, although the magnitude of the transcriptional responses with wortmannin was less than seen in controls. These data strongly suggested that insulin regulated CREB activity through the MAP kinase signaling pathway.

**DISCUSSION**

Insulin is primarily a metabolic hormone that also regulates the proliferation and differentiation of a number of cells and tissues. One of the mechanisms by which insulin regulates transcription is through the phosphorylation and transcriptional activation of CREB in Rat-1A cells, hepatoma cells, preadipocyte, and adipocyte cell lines. Since we observed that insulin enhanced CREB phosphorylation in primary adipocytes and HIRc cell lines (18, 19), a number of groups have seen a similar response to other growth factors including NGF and fibroblast growth factor, via the ERK1/2 and p38 MAPK pathways (4, 24, 39). For these growth factors, both studied in neuronal cell lines, Rsk2 and MAPKAP2, rsk...
family kinases have been implicated in CREB regulation (4, 39). Additionally, PKC, elevated intracellular Ca$^{2+}$, and IgG (via pp70 S6 kinase) can phosphorylate CREB on serine 133. Whether these phosphorylations lead to CREB transactivation appears to depend on cell type and whether the cells are exposed to other factors, such as cAMP agonists simultaneously (40). The importance of the work presented in this paper is the observation that in cell line models of classic, insulin-sensitive tissues, hepatocytes and adipocytes, insulin regulates this ubiquitous transcription factor that has been demonstrated to have a pivotal role in cellular differentiation (24, 40, 41).

We initially observed that insulin led to CREB phosphorylation in primary adipocytes. This was an unexpected finding since insulin's actions classically oppose cAMP which, at that time, was felt to be the primary regulating agent for CREB (21–23, 31). Subsequently, we demonstrated that this enhanced CREB phosphorylation occurred in Rat-1A fibroblasts overexpressing normal human insulin receptors (HIRc cells) (18). The relevance of these observations to CREB transcriptional activity was assessed using multiple promoter constructs including the somatostatin CRE reporter and a truncated PEPCK CRE reporter, each of which would be regulated by endogenous CREB and other CRE binding transcription factors. Also, to assess CREB specifically and exclude other CRE binding transcription factors, the Gal4TKLUC reporter system, a Gal4-CREB responsive transcription system was employed. In this system, either insulin or Bt2cAMP stimulated transcription to a similar extent in HIRc cells. Thus, insulin-mediated CREB phosphorylation appeared to be transcriptionally relevant.

CREB was initially characterized as a substrate of PKA. Activation of PKA by increases in intracellular cAMP results in the increase of CREB transcriptional transactivation activity through the phosphorylation of CREB serine 133 (22, 31). We have shown that the treatment of hepatoma, fibroblast, and adipocyte cells with insulin also results in the phosphorylation of CREB at serine 133. The importance of CREB serine 133 phosphorylation following insulin treatment was further highlighted by experiments in which mutation of serine 133 to alanine completely blocked the insulin responsiveness of a chimeric Gal4-CREB protein in transfection assays. Mutation of other individual CREB serine residues to alanines had no effect on insulin responsiveness of these constructs. These observations were consistent with data from other groups regarding the commonality of serine 133 for CREB transcriptional regulation (21, 33) wherein cAMP stimulation led to enhanced CREB phosphorylation occurred in Rat-1A fibroblasts (22, 31). We hypothesized that the increased CREB phosphorylation specifically blocked by dominant negative CREB in adipocytes. It will be interesting to define the phenotypic consequences of this novel mechanism of insulin-modulated gene expression.

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