Insulin Induction of Apolipoprotein AI, Role of Sp1[†]

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ABSTRACT: Apolipoprotein AI (apo AI) is the major protein component of serum high-density lipoproteins. The abundance of apo AI correlates inversely with the risk of ischemic heart disease (IHD) and thus enhanced expression of the protein is expected to reduce the risk of IHD. Our previous studies show that insulin enhances apo AI promoter activity and this action requires the GC-rich insulin response core element (IRCE, −411 to −404). The motif binds to a ubiquitous transcription factor Sp1. We have extended studies that examine insulin induction of apo AI using a 41 bp (−425 to −385) fragment of apo AI DNA linked to the trout metallothionein TATA box and fused to luciferase (pIRCE-Luc). Luc activity in Hep G2 cells transfected with pIRCE-Luc was stimulated by insulin, an insulin mimetic bisperoxo (1,10-phenanthroline) oxovanadate (bpv) and the phorbol ester (PDBu). Our previous studies showed that insulin action on apo AI gene transcription flowed down two signaling pathways: Ras-raf and PI3K, leading to activation of the MAPK and PKC kinases, respectively. In contrast, PDBu activates only the PKC pathway. Although insulin and PDBu activation of apo AI were distinct, the cascades involved all appeared to target Sp1. Furthermore, exposure of transfected cells to okadaic acid or a phosphatase inhibitor also increased Luc activity and suggested a potential role for phosphorylation, likely involving Sp1. If true, then changes in the IRCE binding activity of Sp1 should be detected following exposure to MAPK, PKC, or the protein phosphatase I (PPI) alone and in various combinations followed by assaying the ability of Sp1 to bind the IRCE. Sp1 binding activity increased with either MAPK or PKC. Although exposure to PPI also affected IRCE binding activity of Sp1, whether it increased or decreased was dependent on the order of exposure to the protein. In summary, the IRCE alone can mediate the stimulatory effects of insulin, bpv, and PDBu, and Sp1 enhances these responses that may arise from phosphorylation of the protein.

Apolipoprotein AI,[1] apo AI is the major protein component of the serum high-density lipoproteins, HDL (*1*, *2*). These particles mediate "reverse cholesterol transport", RCT, a normal physiologic mechanism that shuttles cholesterol from peripheral tissues to the liver for further metabolism and eventual excretion (*3*, *4*). Enhanced RCT lowers total body cholesterol and thus increased abundance of serum apo AI or HDL correlates with a reduced risk of coronary atherosclerosis. Epidemiological and interventional studies have estimated that for each 1% increase in the level of HDL the risk of cardiovascular events is reduced by 2−3% (*5*, *6*). We believe that identifying the molecular mechanisms, which regulate apo AI gene expression will be useful in the development of new therapeutic agents to induce the production apo AI and thereby reduce the incidence of cardiovascular disease.

The regulation of apo AI is complex, requiring interplay of both positive and negative modulators of promoter activity (*7*, *8*). Our recent studies show that insulin stimulates but glucose inhibits the rat apo AI promoter activity in Hep G2 cells (*9*) consistent with an initial report of Karathanasis (*10*). The actions of these two physiological modulators of apo AI gene expression are mediated by a GC-rich insulin response core element, IRCE (−411 to −404). This motif is similar to the insulin response element, IRE, and/or glucose response element of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and fatty acid synthase (FAS) genes (*11*, *12*). Although the sequences of these motifs share similarities, the transcriptional factors that bind to these elements do not. Whereas the GAPDH IRE binds to a protein called IRE-ABP (*11*−*13*), some of the homologous elements within the FAS promoter bind Sp1 (*11*, *14*, *15*). Despite the similarities between the GAPDH and FAS insulin responsive

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[1] Abbreviations: Apo AI, apolipoprotein AI; IRCE, insulin response core element; HDL, high-density lipoprotein.

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motifs, it appears that these elements may interact with at least two different transcription factors.

Sp1, an ubiquitous transcription factor that binds GC-rich motifs, trans-activates promoters via a glutamine rich domain and has an important role in basal transcription of "housekeeping" genes that lack a TATA box (*16*, *17*). But more recent data put Sp1 in the role of a mediator of "cross-talk" between intracellular pathways and regulation of gene transcription. In this report, we have examined insulin induction of apo AI expression. More specifically, we focused our studies on the interaction between Sp1 and the IRCE as a model to further understand the role that it plays in mediating signaling initiated by insulin and agents that activate other intracellular pathways.

EXPERIMENTAL PROCEDURES

Materials and Methods. Cell Lines and Reagents. Human hepatoblastoma cell line, HepG2 (ATCC, Rockville, MD) were grown in Eagle's minimal essential medium (EMEM, Life Technologies Inc.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM Na pyruvate, and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) in a humidified atmosphere of 5% CO_2. Activators and inhibitors of signal transduction pathways were obtained as follows: human insulin; phorbol dibutyrate, PDBu; and okadaic acid were purchased from Sigma (St Louis, MO). Signaling pathway activators or inhibitors, bisperoxo (1,10-phenanthroline) oxovanadate, bpv, and U0126, were purchased from Calbiochem.

Plasmid Constructs. To create the reporter, pIRCE-LUC containing the IRCE fused to luciferase, a synthetic double-stranded oligonucleotide (−425 to −385, gagctcTGCAACGAAAC-TTTGAGGCGGGGATGTGAGTTCAGGAGCCC with an intact 5′ [lowercase letters] but 3′ mutated *SacI* sites to facilitate directional cloning), was inserted into the *SacI* site of rainbow trout metallothionein-TATA luciferase (gift from L Gedamu, U of Calgary, Calgary, AB). The orientation and sequence of the individual clones were confirmed by restriction mapping analysis followed by nucleotide sequencing, respectively. The human Sp1 expression plasmid, CMV-Sp1, was kindly provided by Dr. Robert Tjian, Berkley, CA (*16*, *18*).

Bacterial Expression of GST-Sp1. The coding region of Sp1 cDNA was obtained using RT-PCR. The RNA template for this reaction was total RNA extracted from Hep G2 cells using Trizol reagent (GIBCO-BRL). The forward primer (5′-cgaattcatgagcgaccaagatcactcc-3′) and a reverse primer (5′-cgtcgactcagaagccattgccactgata-3′) with added *EcoRI* and *SalI* flanking the N- and C-terminals (Genbank: AB039286 and J03133) were used in the RT-PCR. The resulting product was inserted into the pBluescript II KS (+) vector (Stratagene) and its identity confirmed by DNA sequencing. The insert was excised by digestion with *EcoRI* plus *SalI* and then subcloned into pGEX 4T-1 (Amersham Pharmacia Biotech) to enable expression of a fusion protein, GST-Sp1. This construct was used to transform BL21:DE3[LysS], and bacteria containing the vector were induced to express GST-Sp1 followed by partial purification using a glutathione column (Amersham Pharmacia Biotech) according to manufacturer's protocol. The elute was dialyzed against 10 mM Tris-HCl, 50 mM NaCl, 10 mM DTT, 0.5 mM EDTA and used in both reactions with kinases or phosphatases and EMSA analysis.

Expression Vector for MEK. The cDNA encoding human MEK-1 and MEK-2 (*19*) were amplified using RT-PCR. The sequences of the primer pairs for MEK-1 were cgggatccaaaatgc-ccaagaagaagc and ggaattcccaaacacttagacgccag and for MEK-2 were ggaattcccctatgggccccggctagag and actgtcacacggcggtgcgcgtggg. The fragments were cloned into the expression vector, pCDNA3.1 (Invitrogen) under the control of CMV promoter at the restriction enzyme sites of *BamHI* to *EcoRI* for MEK1, and *Eco RI* to *EcoRV* sites for MEK2. All inserted fragments were confirmed by sequence analysis.

Stable and Transient Transfection. All plasmid DNAs were purified using Qiagen columns according to manufacturer's directions. Stably transfected Hep G2 cells were created by cotransfection of pIRCE-Luc and pCMV/RC2 (Invitrogen), a plasmid that carried neomycin resistance, into HepG2 cells using LipofectAMINE (Life Technologies Inc.). Transfected cells were selected by adding neomycin, G418 (Life Technologies Inc.), to the culture medium. Distinct, well-isolated colonies were picked and assayed for luciferase activity. Neomycin resistant cells containing pIRCE-Luc were incubated for 16 h in EMEM without FBS before treatment with reagents of interest. Numerous individual clones were selected for luciferase assay studies in order to avoid colony specificity. In transient transfection studies, plasmids of interest were inserted into HepG2 cells using LipofectAMINE as described above. A total of 4.0×10^5 HepG2 cells were seeded per well of six-well plates (Falcon) and grown overnight before adding 0.8 μg of pIRCE-Luc, 0.8 μg of Sp1 expression vector, and 0.5 μg of rous sarcoma virus-β-galactosidase (RSV-β-Gal) plasmid as the internal control and 10 μL of LipofectAMINE in 1 mL of antibiotic/FBS-free EMEM. The expression was normalized against a control of empty expression vector. After 5 h, the cells were washed and incubated with fresh medium for 24 h. Cells were washed and incubated in FBS-free EMEM. After overnight incubation, cells were treated in the presence and absence of reagents for 24 h, harvested, and assayed for luciferase and β-galactosidase activity (see below). Studies that examine U0126, a MEK inhibitor, were added to the cells at a concentration of 5 μM for 4 h prior to exposure to insulin of bpv. In experiments requiring reagents for various signaling pathways, the same volume (less than 0.1% of the total volume of culture media) of carrier (Me_2SO or H_2O) was used as the negative control.

Luciferase/β-Galactosidase Assays. Luciferase, Luc activity in the cells was assessed using a commercial assay kit (Promega). In brief, transfected cells were harvested and resuspended in 250 μL of passive lysis buffer. The supernatant upon centrifugation at 10 000 rpm for 2 min was stored at −70 °C until analysis. Ten μL of this cellular extract was mixed with 50 μL of the luciferase assay buffer (Promega), and the Luc activity was measured as light output (10s) in a Monolight 2010 luminometer (Analytical Luminescence, San Diego, CA). Efficiency of the cell transfection was monitored using β-galactosidase activity and its activity was measured as described previously (*9*). Relative Luc activity of a specific construct was calculated by dividing activity found in control cells transfected with the rainbow

trout metallothionein-TATA luciferase vector without the IRCE (empty vector).

Electrophoretic Mobility Shift Assay. Complementary strands of synthetic DNA duplexes spanning −417 to −385, 5′-ACTTTGAGGCGGGGATGTGAGT-3′ from the apo AI gene, were annealed and radiolabeled at the 5′-ends by incubating with [^{32}P]γ-ATP and T4-polynucleotide kinase (Amersham Pharmacia Biotech). Each binding reaction of 10 μL contained EMSA buffer with the following composition: 10 mM Tris-HCl (pH 8.0), 1 mM ZnCl, 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, 0.5 μg of poly(dI−dC), 0.5 μg of bovine serum albumin, 1 fmol of radiolabeled probe, and 10 μg of Hep G2 cellular extract or 10 pg of recombinant GST-Sp1. Treatment with 0.1 μg of active kinases Erk-2 (Upstate Biotechnology), 25 ng of PKC (Promega), or 2.5 units of protein phosphatase I, PPI (New England Biolabs), was performed by adding the enzyme to Hep G2 nuclear extract or GST-Sp1 in the presence of buffer recommended by the manufacturers. The reactions were incubated at 30 °C for 30 min followed by adjustments to the reaction such that it equaled the EMSA-buffer before adding radiolabeled probe. The mixture was incubated for an additional 20 min at room temperature and then analyzed using EMSA.

For studies involving sequential addition PPI and kinase or the converse, the first enzyme was added to GST-Sp1 15 min prior to the second enzyme followed by an additional 15 min of incubation before adding the radiolabeled IRCE. In competition analysis, 50-fold molar excess of unlabeled homologous or nonhomologous competitor DNA was added to the reaction prior to the addition of nuclear extracts. All reactions were separated on a 4% polyacrylamide nondenaturing gel buffered with TBE. Electrophoresis was performed at 200 V for 2−3 h at 4 °C followed by drying of the gel and autoradiography with Kodak XAR-5 film.

In Vitro Phosphorylation of Sp1. Erk-2 and PKC phosphorylation of GST-Sp1 was carried out according to conditions recommended by the manufacturers at 30 °C for 30 min in the presence of 1 μCi [γ-^{32}P]ATP. For the use of PPI, GST-Sp1 protein was first phosphorylated at 30 °C for 15min prior to adjustment of reaction conditions to suit PPI activity followed by 15 min incubation. The reaction products were analyzed using EMSA and the protein was separated using 7.5% SDS-gel followed by transfer to PVDF membrane and signal detected using autoradiography. After autoradiography exposure, the membrane was washed and probed by Sp1 antibody (Geneka Biotechnology Inc.), followed by horseradish peroxidase-conjugated anti-rabbit antibody (1/5000 dilution). The signal was detected using an ECL kit (Amersham Pharmacia Biotech) to visualize bound primary antibody.

Statistical Analysis. Statistical comparisons were made by one-way analysis of variance and Student's *t*-test, with $P < 0.01$ or $P < 0.05$ considered significant.

RESULTS

Activity of pIRCE-Luc. Our previous studies showed that insulin induction of apo AI expression in Hep G2 cells required a cis-acting element the IRCE (*9*). This GC-rich motif is bound by a ubiquitous transcription factor, Sp1 (*20*). The presence of multiple putative Sp1-like GC-rich motifs in the rat apo AI promoter raises the question of whether a single copy of the IRCE can mediate the actions of insulin (*21*). Therefore, we addressed this question using 41 bp fragment (−425 to −385) of the promoter that has embedded within it the IRCE. This fragment was placed in front of the rainbow trout metallothionein TATA motif and then fused to the luciferase reporter gene to yield pIRCE-Luc (Figure 1A). In the absence of the IRCE motif, the metallothionein TATA was not responsive to insulin, bpv or PDBu (data not shown). The activity of pIRCE-Luc was tested in Hep G2 cells transfected either transiently (data not shown) or stably (Figure 1B,C). Luciferase, Luc, activity was minimal in Hep G2 cells transiently or stably transfected with empty vector (pGL3 only), but in cells containing pIRCE-Luc, the Luc activity was easily detected. Several of the stably transfected cells were tested and found to have detectable Luc activity of comparable level. These results show that both transiently or stably transfected Hep G2 cells containing pIRCE-Luc provide a useful tool for addressing the above question.

IRCE Alone Is Sufficient to Mediate Insulin Action. Whether the IRCE in isolation could mediate the effects of insulin and other agents was tested in stably transfected Hep G2 cells by exposure to 100 μU/mL insulin for 24 h. Luc activity increased 1.8-fold in treated compared to the untreated control cells (Figure 1B). This induction was similar to the previously observed 1.9±0.1-fold induction of the full-length (−474 to −7) promoter in transiently transfected cells (*9*). In addition, insulin induction of promoter activity was matched by a 1.9-fold rise in abundance of apo AI protein in the spent media (Figure 1E). Not surprisingly, the insulin mimetic, bpv, also induced Luc activity by 3.6-fold in transiently (data not shown) or stably (Figure 1B) transfected cells. This induction of promoter activity caused a 2.2-fold rise in abundance of apo AI protein (Figure 1E).

Previous studies (*20*) showed that insulin induction of apo AI gene transcription was split equally between two signaling cascades: the phospholipase Cγ requiring PKC and the Ras-Raf involving the MAPK pathways. Whether pIRCE-Luc could mediate the actions of PKC was tested in stably transfected cells exposed to the phorbol ester, PDBu. Luc activity in these cells increased 3.0-fold compared to control. Both bpv and PDBu induction of Luc activity was dose-dependent (Figure 1C,D).

Last, we tested effects of hyperglycemia on activity of pIRCE-Luc. Unexpectedly, ability of high glucose to inhibit apo AI expression observed using the full-length promoter was not evident following exposure of the stably (Figure 1B) or transiently transfected cells to 22.5 mM of either D- or L-isomer of glucose (*9*). Together, these results show that the IRCE alone has the ability to mediate the stimulatory actions of insulin, bpv, and PDBu but not the inhibitory effects of D-glucose or L-isomer of glucose.

Induction of IRCE Activity by Sp1 but not by Sp3 Overexpression. Our previous studies show that the IRCE binds Sp1 and this factor enhances activity of the motif in the context of the full-length promoter. This finding prompted us to measure Luc activity in cells cotransfected with pIRCE-Luc and a vector that overexpressed Sp1. In these cells, the activity of pIRCE-Luc was 5.9±0.1-fold higher versus that transfected with pIRCE-Luc plus empty vector (Figure 2A,

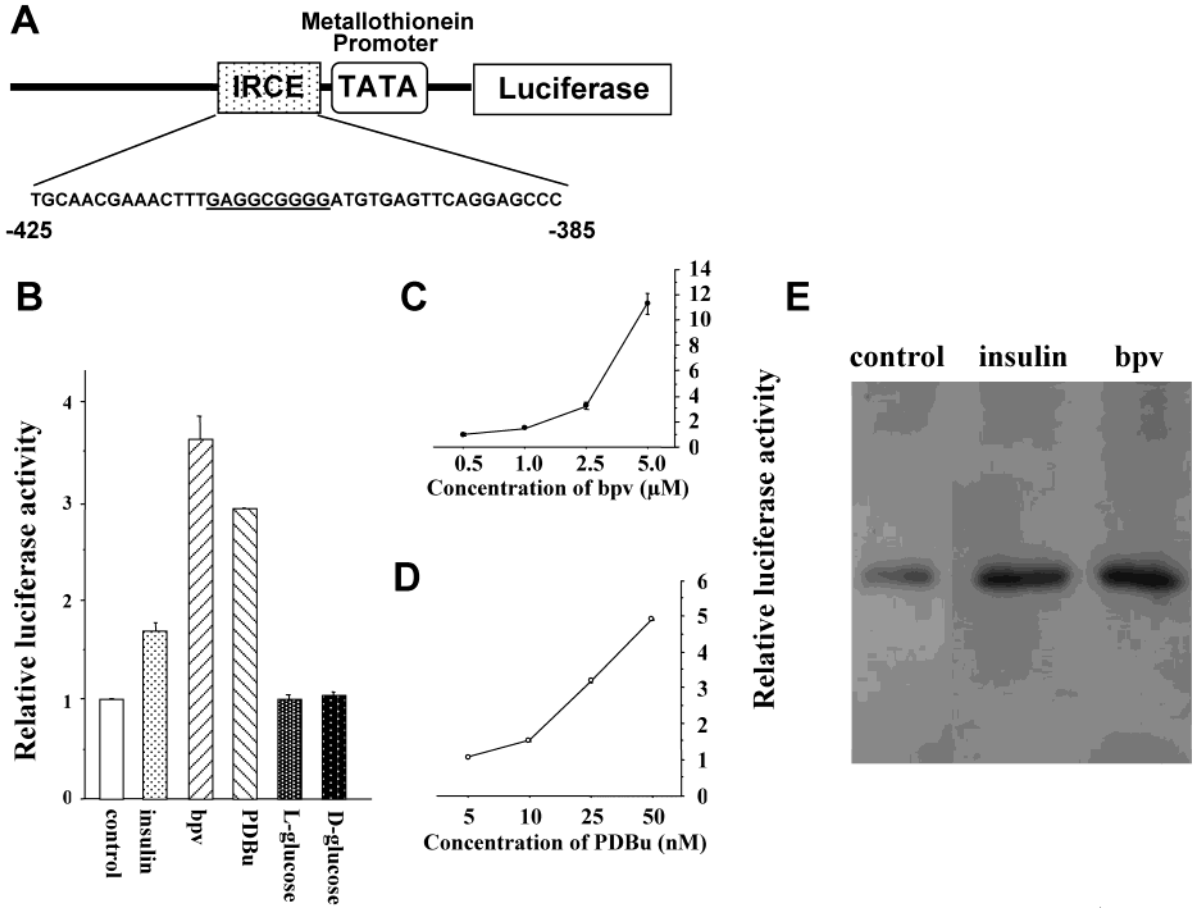

FIGURE 1: Schematic of pIRCE-Luc construct and its activity in Hep G2 cells. (A) The pIRCE-Luc construct containing rat apo AI DNA (−425 to −385, the Sp1 binding sequence is underlined) placed 5′ of the rainbow trout metallothionein TATA box and then fused to the reporter gene, luciferase. (B) Luc activity in Hep G2 cells stably transfected with pIRCE-Luc and then exposed to control media or that containing 100 μU/mL insulin, 2.5 μM bpv, 25 nM PDBu, 22.5 mM L-glucose or 22.5 mM D-glucose for 24 h. Panels C and D show Luc activity in Hep G2 cells containing pIRCE-Luc in response to increasing concentrations of bpv and PDBu for 24 h. Panel E contains a Western-blot showing signal from apo AI protein in spent media from Hep G2 cells treated for 48 h with control media or that containing 100 μU/mL insulin, 2.5 μM bpv.

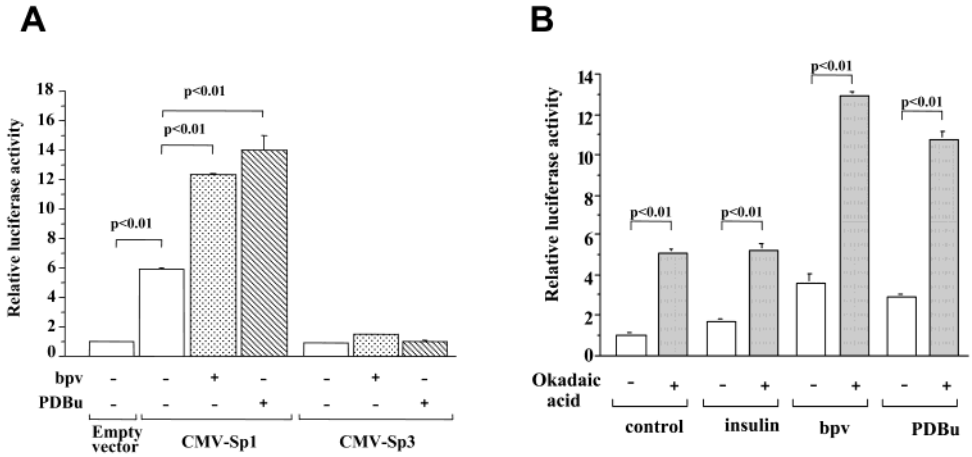

FIGURE 2: Overexpression of Sp1 but not Sp3 and okadaic acid augments activity of IRCE. (A) Luc activity in Hep G2 cells Stably containing pIRCE-Luc and then transiently transfected with CMV-Sp1 or -Sp3 and then exposed to control media or that containing 100 μU/mL insulin, 2.5 μM bpv, or 25 nM PDBu for 24 h. Each bar shows the mean and S.E.M. ($n \geq 3$). Where the error bars are not seen, the S.E.M. is subsumed within the mean. (B) Luc activity in the stably transfected cells in the absence (−) or presence of (+) 10 nM okadaic acid and then exposed to control media or that containing 100 μU/mL insulin, 2.5 μM bpv, or 25 nM PDBu for 24 h. Each bar shows the mean and S.E.M. ($n \geq 3$).

columns 1 and 2). When compared to previous results using the full-length apo AI promoter, Luc activity was only 1.7 ± 0.3-fold higher in the presence of overexpression of Sp1 (*20*). The addition of bpv or PDBu to the cotransfected cells yielded a 12- and 14.0 ± 1.0-fold increase in Luc activity, respectively. In studies that overexpressed Sp3 (Figure 2A) another member of the Sp1 transcription factor family, this factor did not enhance activity of pIRCE-Luc. Together, these

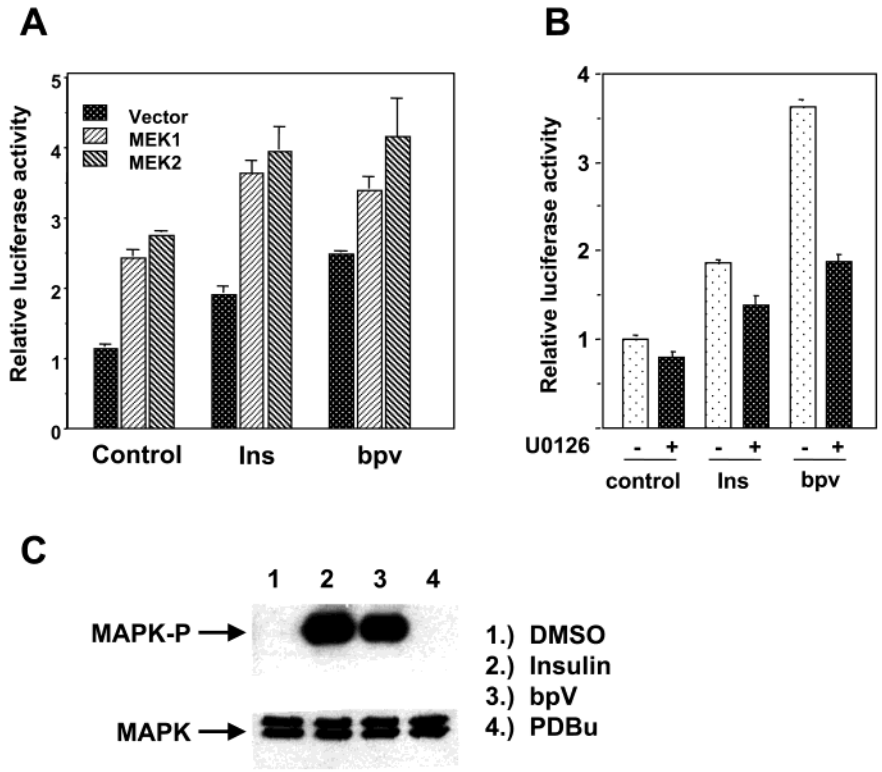

FIGURE 3: Insulin and bpv action are mediated by MEK-1/-2 and MAPK. Panel A shows Luc activity of pIRCE-Luc in Hep G2 cells without or with cotransfection of a vector that enables expression of MEK-1 or MEK-2 followed by exposure to control media or that containing 100 μU/mL insulin or 2.5 μM bpv for 24 h. Panel B shows the effects of Hep G2 cells transfected with pIRCE-Luc without and with prior treatment using 5 μM of the MEK inhibitor, U0126 followed exposure to control media or that containing 100 μU/mL insulin or 2.5 μM bpv. (C) The upper panel contains a Western blot probed with an antibody against Phospho-p44/42 MAPK monoclonal antibody (cell signaling). The lower panel was probed with antibody against MAPK polyclonal antibody (CalBiochem). Hep G2 cell lysate treated with DMSO (control) or that containing various agents for 24 h. The contents of the lanes are as follows: 1, DMSO control; 2, 100 μU/mL insulin; 3, 2.5 μM bpv; and 4, 50 nM PDBu.

results indicate that overexpression of Sp1 but not Sp3 enhances basal and induced activity of the IRCE.

Okadaic Acid Augments Stimulation by Insulin, bpv, and PDBu. The actions of insulin, bpv, and PDBu are mediated by intracellular signaling with participation of kinase(s). If so, then the effect of okadaic acid, an inhibitor of serine/threonine phosphatase activity, should augment the actions of these agents (*22*). Therefore, okadaic acid was added to cells stably transfected with pIRCE-Luc prior to adding insulin, bpv, or PDBu. Results (Figure 2B) showed that okadaic acid augmented the insulin, bpv, and PDBu induction of pIRCE-Luc by 2.9-, 3.7-, and 3.6-fold, respectively, compared to untreated cells. Similar findings were present in transiently transfected cells (data not shown). These results suggest that phosphorylation of cellular protein(s) plays an important role in the insulin, bpv, and PDBu stimulation of pIRCE-Luc activity.

Inhibition of MEK and PKC Pathways Block Induction Mediated by IRCE. Insulin induction of apo AI in Hep G2 cells require the participation of the Ras-Raf, MAPK pathway because dominant negative Ras blocks, in part, the actions of the hormone (*23*). To define intermediates downstream of Ras, we tested whether insulin action required two known MEK kinase isoforms. Therefore, the cDNAs encoding the human MEK-1 or -2 kinase isoforms were cloned using RT-PCR and inserted into pcDNA for expression in Hep G2 cells. Cotransfection of pIRCE-luc with either the MEK-1 or MEK-2 enhanced Luc activity to comparable levels (Figure 3A, left set of columns). Insulin or bpv treatment of cells cotransfected with the reporter and MEK-1 or MEK-2 also increased Luc activity (Figure 3A, middle and right set of columns). These studies of MEK-1 and −2 simply show that both kinases have the ability to enhance IRCE activity.

Next, cells transfected with pIRCE-Luc were treated with and without 5 μM U0126, an inhibitor of MEK kinase. This inhibitor decreased basal activity of the reporter construct and in the presence of insulin or bpv; U0126 attenuated but did not block completely actions of either agent (Figure 3B). The partial inhibition is consistent with the division of insulin or bpv action via two signaling pathways (*23*), one of which requires the MEK kinase(s). The observations gathered using Okadaic augments that of the preceding section and points to the participation of both MEK-1 and -2 in mediating insulin and bpv induction of IRCE activity.

Participation of Activated MAPK in Insulin and bpv Action. The preceding findings suggest a role for MAPK, the next component in this signaling cascade. Therefore, we probed for the presence of activated MAPK in cells exposed to insulin or bpv using a phospho-specific MAPK antibody in Western blot analysis of cell lysate. Results (Figure 3C) showed that whereas activated MAPK was found in cells treated with insulin or bpv (Figure 3C, lanes 2 and 3), the level of the active kinase was not detected in control cells or those treated with the PKC activator PDBu (Figure 3C, lanes 1 and 4, respectively). That equal amounts of protein were loaded into each lane was demonstrated using an antibody recognized MAPK and not just the phosphorylated form. The abundance of MAPK was the same in all samples

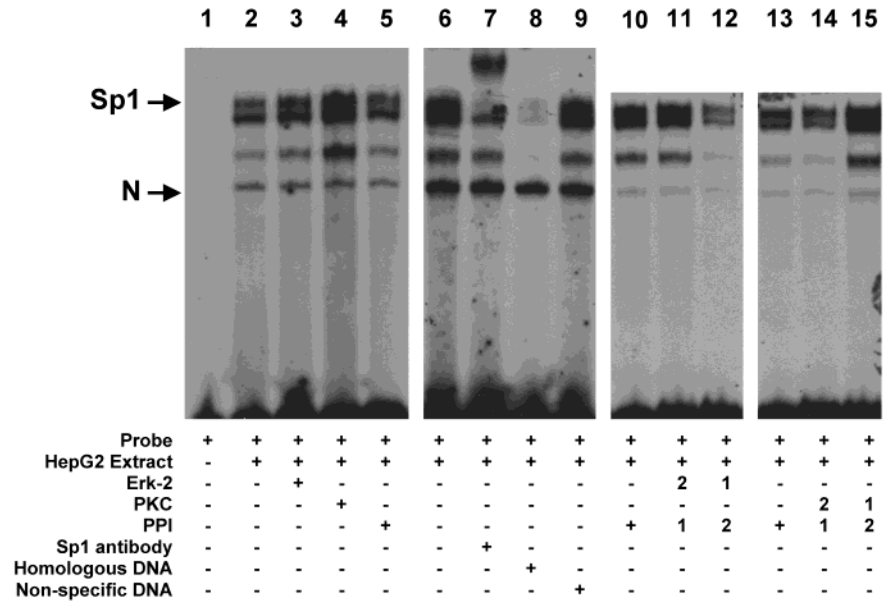

FIGURE 4: Kinase and PPI treatment of Sp1 extracted from Hep G2 cells. Results of EMSA studies yielded autoradiograms shown here. All reactions contained nSp1 extracted from Hep G2 cells except for lane 1, radiolabeled probe alone. The contents all other lanes that contain nSp1 are treated with the following: 2, no treatment; 3, Erk-2; 4, PKC; 5, PPI; 6, no treatment; 7, Sp1 antibody; 8, 50-fold molar excess of homologous competitor; 9, 50-fold molar excess of nonhomologous competitor; 10, PPI; 11, PPI followed by Erk-2; 12, Erk-2 followed by PPI; 13, PPI; 14, PPI followed by PKC; and 15, PKC followed by PPI.

(Figure 3C, lower panel). The findings in this and the preceding section confirm the participation of MEK plus activated MAPK in a signaling pathway activated by insulin and bpv but not PDBu.

Sp1 Phosphorylation and Binding to IRCE. Insulin and bpv induction of apo AI is mediated by MEK and MAPK (*23*). Activation of these kinases is known to alter the phosphorylation status of specific proteins. Since Sp1 may mediate cross-talk between signaling and gene regulation, perhaps intracellular pathways affect Sp1 phosphorylation and thus modulates apo AI gene transcription. Studies to date have identified Ras-raf, MEK-1/2, MAPK, Sp1, and IRCE to be sequential components of a cascade by which insulin/bpv induce apo AI. Whereas the serial activation of Ras-raf followed by MEK-1/2 and MAPK is clear, how Sp1 and its recognition motif, the IRCE, fit into the model is not. Since Sp1 is a phosphorylated protein (*24*), we postulated that components of the signaling cascade alter the phosphorylation of Sp1 and thus modulates its IRCE binding activity. We have examined this question by measuring the IRCE binding activity of Sp1 from two sources: (a) that extracted from Hep G2 cells and (b) bacterially expressed.

First, we measured IRCE binding activity in Hep G2 cells using EMSA analysis (Figure 4). An autoradiograph of the gel revealed the presence of four protein:IRCE complexes. The Sp1 extracted from Hep G2 cells will be called native Sp1 (nSp1). When nSp1 is bound to the IRCE, it formed a complex nSp1:IRCE with the slowest electrophoretic mobility (Figure 4, lanes 2). This complex contained nSp1 because the addition of a monoclonal antibody against Sp1 caused a supershift (Figure 4, lane 7). Furthermore, the presence of a 50-fold molar excess of homologous abolished formation of nSp1:IRCE but not nonspecific competitor DNA (Figure 4, lanes 8 and 9, respectively). In addition, the two complexes that migrated faster than Sp1:IRCE were also displaced by homologous competitor DNA, thus suggesting that these were also IRCE binding proteins but not Sp1. The fourth complex (Figure 4, designated N) is not specific because neither homologous nor nonhomologous competitor affected abundance of the fastest migrating band.

Whether MAPK could directly phosphorylate nSp1 was tested by incubating Hep G2 extract with Erk-2, an isoform of MAPK. This reaction caused a slight increase in the binding of nSp1 to the IRCE (Figure 4, compare lanes 3 and 2). Next we tested the ability of PKC to modulate nSp1 binding to the IRCE. Exposure of Hep G2 extract to PKC caused a marked increase in nSp1 binding to the IRCE (Figure 4, lane 4). If kinases enhance Sp1 binding to the IRCE, then treatment with the protein phosphatase I, PPI, a Ser/Thr specific phosphatase, should lower this activity. Therefore, we exposed Hep G2 extract to PPI alone, but this had no effect on nSp1 binding to the IRCE (Figure 4, lanes 5, 10, and 13).

The above studies showed that when nSp1 was treated with Erk-2, PKC, or PPI, only the kinases affected IRCE binding activity. In the cell, differential activity of separate signaling pathways will expose nSp1 to kinase first and then phosphatase or the reverse. To better mimic conditions in the cells, we treated nSp1 with PPI first followed by Erk-2 (Figure 4, lane 11) and noted no change in binding activity (Figure 4, lane 10 compared to 11). Unexpectedly, when Sp1 was treated with Erk-2 first and then PPI, the binding to the IRCE was decreased significantly (Figure 4, compare lanes 10 and 12). In parallel studies where Erk-2 was replaced by PKC, prior treatment of Hep G2 extract with PPI first and then PKC had no effect on nSp1 binding to the IRCE (Figure 4, compare lanes 14 and 13). However, when PKC was used first and then PPI, it caused a marked increase in nSp1 binding to the IRCE (Figure 4, compare lanes 13 and 15).

Next the above studies were repeated by replacing Hep G2 extract with bacterially expressed Sp1 because bacterial proteins have not been exposed to modifying enzymes found

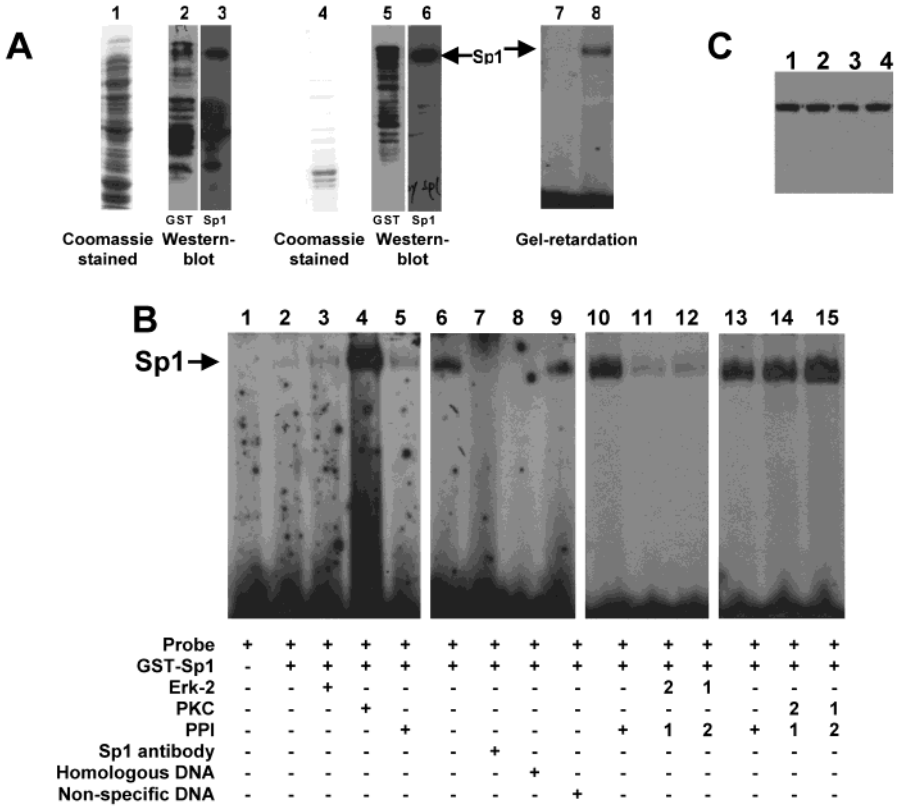

FIGURE 5: Bacterially expressed Sp1 treated with kinase and PPI. Panel A shows analysis of bacterial expression of the fusion protein, GST-Sp1. Lane 1 shows coomassie blue staining of a SDS−PAGE gel containing crude lysate from bacteria transformed with GST-Sp1 expression vector. Lanes 2 and 3 show Western blot analyses of the extract probed with either antibody against GST or Sp1 in lanes 2 and 3, respectively. Next the lysate was subjected to affinity column chromatography that binds the GST-moiety of the fusion protein and the resulting eluate subjected to repeat analysis and EMSA analysis for binding to radiolabeled IRCE. Lane 4 shows coomassie-blue staining of a SDS-PAGE gel containing eluate from the affinity column. Lanes 5 and 6 show Western blot analyses of eluate probed with either antibody against GST or Sp1 in lanes 5 and 6, respectively. The high abundance of GST signal in lane 5 may be due to degradation of the fusion protein or specificity of the antibody. An autoradiograph of an EMSA study is presented in lanes 7 and 8, containing free probe or that bound to GST-Sp1, respectively. (B) Results of EMSA studies using GST-Sp1 yielded autoradiograms shown here. All reactions contained GST-Sp1 extracted from Hep G2 cells except for lane 1, radiolabeled probe alone. The contents all other lanes that contain GST-Sp1 are treated with the following: 2, no treatment; 3, Erk-2; 4, PKC; 5, PPI; 6, no treatment; 7, Sp1 antibody; 8, 50-fold M excess of homologous competitor; 9, 50-fold M excess of nonhomologous competitor; 10, PPI; 11, PPI followed by Erk-2; 12, Erk-2 followed by PPI; 13, PPI; 14, PPI followed by PKC; and 15, PKC followed by PPI. Panel C shows a Western-blot containing reactions of GST-Sp1 untreated (lane 1), or treated with Erk-2 (lane 2), PKC (lane 3), or PPI (lane 4).

in mammalian cells. Crude lysate (Figure 5A) from *Escherichia coli* transformed with a Sp1-GST expressing vector or eluate from lysate bound to a GST-affinity column was probed with GST or Sp1 specific antibodies (Figure 5 lanes 1−6). In crude lysate, GST and Sp1 antibodies recognized many species (Figure 5, lanes 1−3), but eluate from the column was different (Figure 5, lanes 4−6). The GST antibody recognized many species in the eluate (Figure 5A, lane 5), but only one major band cross-reacted with the Sp1 antibody (Figure 5A, lane 6).

Gel-retardation analysis of the Sp1-GST enriched eluate (Figure 5A, lane 8) showed that it bound to the radiolabeled IRCE to form a single band. The addition of Sp1 antibody to the EMSA reaction caused a supershift in the Sp1-IRCE protein:DNA complex (Figure 5B, lane 7). That this binding is specific was demonstrated by the ability of 50-fold molar excess of homologous unlabeled IRCE but not nonspecific DNA to displace binding (Figure 5B, lanes 8 and 9, respectively) to the radiolabeled probe. Together, these studies show that bacterially expressed Sp1 binds to the IRCE and this binding is specific.

When Sp1-GST was incubated with the MAPK isoform Erk-2 or PKC, both kinases increased Sp1 binding activity to the IRCE (Figure 5B, compare lanes 3 and 4, respectively with 2). PKC caused a much bigger increase compared to Erk-2. Next we treated the Sp1-GST with PPI (Figure 5B, lane 5) and noted a slight increase in IRCE binding activity. These findings showed that the activities of Erk-2, PKC and PPI all caused an increase in GST-Sp1 binding to the IRCE.

On the basis of the latter observation, Sp1 exposed to any combination of the two kinases and PPI should augment its IRCE-binding activity more than either one alone. Unexpectedly, the treatment of Sp1-GST with PPI first followed by Erk-2 decreased Sp1 binding to the IRCE (Figure 5B, lane 11). This sequential treatment caused IRCE binding activity of GST-Sp1 to fall below the level observed following PPI treatment alone (Figure 5B, compare lanes 10 and 11). A similar finding was evident when Sp1-GST was exposed to Erk-2 first followed by PPI (Figure 5B, compare lanes 10 and 12). This abrogation of activity is not due to protein degradation because a Western blot showed no change in abundance of the intact protein (Figure 5C).

The preceding studies were repeated by substituting PKC for Erk-2 (Figure 5B, lanes 13−15). Results showed that Sp1-GST treated with PPI first followed by PKC had

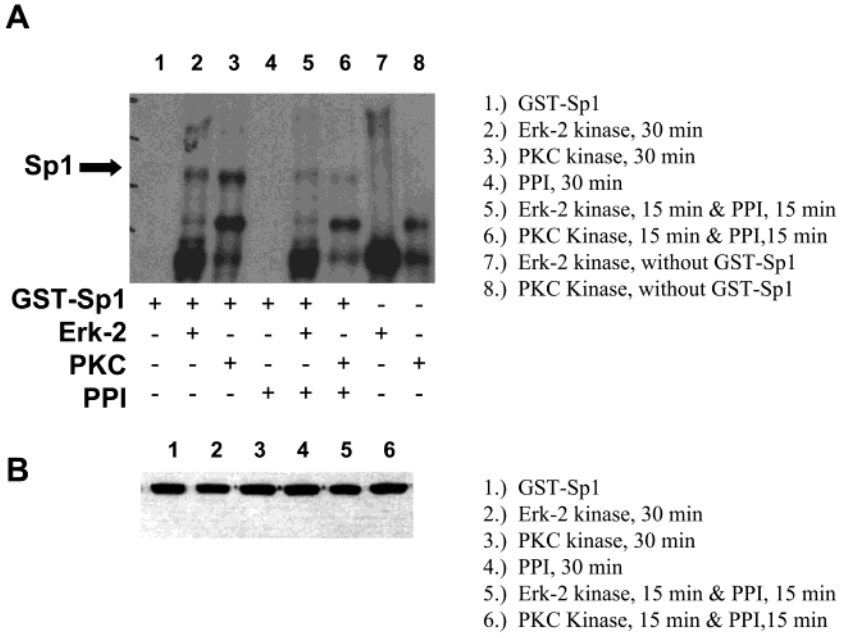

FIGURE 6: In vitro phosphorylation of Sp1. Panel A shows an autoradiograph of an SDS−PAGE separation of proteins and then transferred to PDVF membrane prior to exposing to X-ray film. The study shows the ability of various kinases and PPI to alter phosphorylation of GST-Sp1. The contents are as follows: lane 1, GST-Sp1 exposed to [γ-^{32}P] ATP without kinase; lane 2, GST-Sp1 + [γ-^{32}P] ATP and Erk-2; lane 3, GST-Sp1 + [γ-^{32}P] ATP and PKC; lane 4, GST-Sp1 + [γ-^{32}P] ATP and PPI; lane 5, GST-Sp1 + [γ-^{32}P] ATP and exposed to Erk-2 prior to PPI; lane 6, GST-Sp1 + [γ-^{32}P] ATP and exposed to PKC prior to PPI; lane 7, Erk-2 + [γ-^{32}P] ATP; and lane 8, PKC + [γ-^{32}P]ATP. Panel B shows a Western-blot of the same PVDF probed with Sp1 antibody.

minimal effect on binding of Sp1-GST to the IRCE that was different from PPI alone (Figure 5B, lanes 13 and 14, respectively). In contrast, reversing the order of treatment using PKC first and then PPI (Figure 5B, lane 15) lead to a marked increase in Sp1-GST binding to the IRCE. All studies shown here are representative of 3 separate studies that yielded the same results in terms of their changes in IRCE binding activity.

Although the studies in this section are relatively simple, there are two important findings to highlight: (i) altering phosphorylation state may increase or decrease Sp1 ability to bind to the IRCE; (ii) the apparent order of Sp1 exposure to either kinase or phosphatase will increase or decrease its binding to the IRCE.

Erk-2, PKC or PPI Modulated Phosphorylation of Sp1. The above studies presume that exposure of GST-Sp1 to Erk-2, PKC, or PPI changes phosphorylation of Sp1. To determine changes in the phosphorylation state of Sp1, we used the radioactively labeled [γ-^{32}P] ATP follow activity of the various enzymes. GST-Sp1 was exposed Erk-2 or PKC in the presence of [γ-^{32}P] ATP, and the resulting products were separated on an SDS−PAGE gel and then transferred to PVDF membrane. The radiolabeled products were detected by autoradiography. Results (Figure 6A) showed that GST-Sp1 incubated with [γ-^{32}P] ATP had no detectable radioactive products. This finding indicates that GST-Sp1 does not undergo autophosphorylation.

When GST-Sp1 was incubated with Erk-2 or PKC and [γ-^{32}P] ATP (Figure 6A, lanes 2 and 3, respectively), distinct bands appeared. These bands were absent at a corresponding position in control reactions containing only Erk-2 or PKC and [γ-^{32}P] ATP (Figure 6A, lanes 7 and 8) used to detect autophosphorylation products. Whether PPI could remove phosphates from GST-Sp1 required a prior step of producing phosphorylated Sp1 using Erk-2 or PKC (Figure 6A, lanes 2 and 3, respectively). As expected, no phosphorylated Sp1 appeared in the reaction containing only GST-Sp1, [γ-^{32}P] ATP, and PPI (lane 4). However, when phosphorylated GST-Sp1 was treated with PPI, there was a marked decrease in abundance of radioactivity associated with GST-Sp1 (Figure 6A, compare lanes 5 and 6 with 2 and 3, respectively). These changes were not due to uneven sample loading; the membrane was probed with Sp1 antibody. Results (Figure 6B) showed a similar abundance of Sp1 in all lanes. The fact that GST-Sp1 is a fused protein raised the question of whether phosphorylation was attached to the GST or Sp1 moiety. This question was addressed by digesting the phosphorylated GST-Sp1 with thrombin to divide the fusion protein into its Sp1 and GST components. The digestion products were separated by SDS−PAGE and the radiolabeled product located by radioactivity. Results (data not shown) showed that radioactivity was associated with the Sp1 but not GST. The studies in this section show that activities of Erk-2 or PKC and PPI altered Sp1 phosphorylation. Whereas, the two kinases increase, the PPI decreases phosphorylation of Sp1.

DISCUSSION

Our interest to define the mechanism(s) that induce(s) expression of the apo AI gene underlies the rationale for the present studies. We have previously shown that insulin induction of apo AI expression is mediated by a GC-rich motif the IRCE (−411 to −404). This element interacts with the transcription factor Sp1. More in depth knowledge of the mechanism(s) by which insulin controls apo AI gene activity will improve our understanding of why diabetics have lower levels of apo AI/HDL (*10*). This complication of diabetes mellitus adds to the patient's risk of ischemic

heart disease, which is 2−4-fold higher than that in the normal population (*3*, *25*).

A focused view of IRCE function was possible through use of the reporter construct pIRCE-Luc. This reporter contained a 41 bp of rat apo AI promoter (−425 to −385) that had embedded within it, the IRCE. Activity of pIRCE-Luc reflected actions of various agents on the motif. Exposure of Hep G2 cells containing pIRCE-Luc to insulin, the insulin mimetic bpv, and PDBu increased transcription by 1.8-, 3.6-, and 3.0-fold, respectively. Furthermore, the actions of these agents were amplified in the presence of Sp1 overexpression but not Sp3. Exposure of cells transfected with pIRCE-Luc to the phosphatase inhibitor, okadaic acid with insulin, bpv, and PDBu further augmented Luc activity to higher values of 5.3-, 13-, and 10.8-fold, respectively. The important points arising from these studies are that (i) the IRCE motif alone appears sufficient to mediate the stimulatory effects of all three agents, (ii) Sp1 and not Sp3 augments the actions of the agents, and (iii) phosphorylation may play a role in mediating the actions of the agents.

The exposure of Hep G2 cells to insulin, bpv, or PDBu triggers selected signaling cascades prior to the induction of apo AI expression. The common feature of all three agents is that they eventually lead to the target Sp1. It is presumed that the signaling components somehow alter Sp1 activity in such a way that it enhances ability of the IRCE to increase apo AI gene transcription. Studies mentioned in the preceding paragraph lists agents that initiate the induction process, which require the participation of Sp1 and IRCE. However, the chain of events following exposure to the agents and ending in altered Sp1 activity remains undefined. Our previous studies of this model system (*23*) showed that insulin induction of apo AI required the equal participation of the Ras-raf and PI3K pathways, two cascades that are distinct signaling pathways. Whereas activation of the Ras-raf route increases phosphorylation activity of MAPK, the PI3K pathway enhances activity of PKC. This observation prompted us to ask whether other components of the Ras-raf pathway participated in the insulin or bpv induction of the apo AI gene. This interest underlies the studies that examined MEK-1 and -2, two intermediary kinases in the Ras-raf pathway (*19*). Either isoform in cotransfection experiments augmented the stimulatory actions of insulin and bpv. MEK-1/-2 stimulated the phosphorylation activity of the next component in the cascade, MAPK. Consistent with this fact, activated MAPK was present only in cells treated with insulin or bpv. As expected, activated MAPK was not found in cells exposed to the phorbol ester, PDBu. These observations show the known components of the Ras-raf pathway mediate not only the actions of insulin but also its mimetic, bpv.

Sp1 is a ubiquitous transcription factor. How is it able to effect hormonal and signaling pathway control of a "non-housekeeping" gene such as apo AI? The ability of okadaic acid to enhance insulin, bpv and PDBu stimulation of apo AI expression suggests the participation of protein phosphorylation. Since Sp1 is known to be a phospho-protein (*24*), perhaps the IRCE role in the regulation of apo AI transcription is augmented and modulated by changes in Sp1 phosphorylation. Sp1 is believed to be a target for various kinases. For example, signal transduction pathways triggered by cAMP-dependent protein kinase, PKA, mitogen-activated protein kinase, MAP, Ca^{2+}/calmodulin kinases, CamK, protein kinase C, PKC, and Erk-2 can modulate the activity of Sp1 (*26*−*32*). This is in agreement with the idea that phosphorylation and other post-translational modifications of Sp1 alter its binding and transcriptional activity (*24*, *33*).

Whether activated MAPK or PKC may directly alter Sp1 activity or indirectly via another set of intermediates is not known. To examine the first possibility, we tested the ability of an isoform of MAPK, Erk-2, and PKC to alter the IRCE binding activity of Sp1. If treatment with either Erk-2 or PKC alters the IRCE binding activity of Sp1, this finding suggests a direct route by which these kinases may affect Sp1 activity. Our findings (Figures 4 and 5) show that treatment with either kinase increases Sp1 binding to the IRCE regardless of whether the Sp1 was nSp1 or GST-Sp1 from Hep G2 cells and bacterial expression, respectively. These findings suggest that increased phosphorylation of Sp1 augments its ability to bind the IRCE to enhance apo AI expression.

A logical prediction from the actions of the kinases is that treatment of Sp1 with PPI alone should cause it to lose or decrease its binding to the IRCE. In contrast, we found that IRCE binding activity of either nSp1 or GST-Sp1 exposed to PPI was not different or slightly different, respectively from the controls. Plausible explanations include that both nSp1 and GST-Sp1 were not phosphorylated in Hep G2 or bacteria. This is highly unlikely for nSp1 because inside the cell it is exposed to numerous kinases and initial studies that identified it to be a phospho-protein used material extracted from cells (*24*). That GST-Sp1 produced in bacteria may also be phosphorylated comes from studies that show the phosphorylation of other mammalian proteins made in *E. coli* (*34*, *35*). The inability of PPI to alter the IRCE binding activity of nSp1 or GST-Sp1 may arise from the fact that the sites of Sp1 phosphorylation were not accessible to PPI under the conditions of the assay. In addition, this finding stands in contrast to reports in the literature where dephosphorylation is reported to increase the DNA binding activity of Sp1 (*36*, *37*). One potential explanation for why our observation differs from that of the literature is perhaps dephosphorylation of Sp1 leading to increases in DNA binding is sequence specific and this feature does not include the IRCE motif.

In the cell, Sp1 is exposed to both kinases and phosphatases. To recreate conditions that mimicked more closely intracellular conditions we performed sequential treatment of Sp1 with either kinase followed by PPI or the converse. When nSp1 was treated with PPI first and then Erk-2, its binding activity to the IRCE was not different from that of untreated or exposure to PPI alone. However, the reverse order of treatment caused a significant decrease in nSp1 IRCE binding activity. The repetition of these studies in which Erk-2 was replaced with PKC showed that exposure to PPI first followed by PKC yielded a slight decrease in the binding of nSp1 to the IRCE. The inverse order of treatment, i.e., PKC followed by PPI, caused a marked increase in nSp1 binding to the motif. This may be due to changes in conformation of Sp1 resulting from PKC activity exposing sites to PPI and the activity of PPI increases IRCE binding activity of Sp1.

The same studies in the preceding section were performed using GST-Sp1 and yielded results that paralleled closely

the observations noted for nSp1. The major difference being that treatment of GST-Sp1 with PPI followed by Erk-2 caused a marked drop but studies of nSp1 using this protocol did not change IRCE binding activity (Figures 4 and 5B, compare lane 11 from each figure). One potential explanation is that PPI caused a change in conformation of GST-Sp1 such that the phosphorylation site(s) became inaccessible to Erk-2. Exposure of GST-Sp1 to Erk-2 first and then PPI also decreased its binding to the IRCE. This finding mimicked that of nSp1 treated in a similar fashion (Figures 4 and 5A, lane 12 in both). The use of PKC rather than Erk-2 in the studies to treat GST-Sp1 yielded findings that matched closely that of nSp1 in that PPI first and then PKC did not alter IRCE binding significantly but the reverse order of treatment increased GST-Sp1 binding to the IRCE. This latter observation is consistent with the findings related to Sp1 binding to the acetyl CoA carboxylase, aldose reductase and pyruvate kinase M2 promoter is enhanced by dephosphorylation (*36*, *37*).

The outcome of these studies showed that treatment with either kinase alone caused an increase in the binding of Sp1 to the IRCE. However, in combined treatments with kinase and PPI, the IRCE-binding activity of Sp1 was distinct. Whereas treatment with PPI first followed by Erk-2 did not affect the binding of nSp1 to the IRCE, exposure to Erk-2 first and then PPI caused a significant decrease in binding to the IRCE. The exact opposite was observed for PKC: when nSp1 was exposed to PKC first and then PPI, there was a marked increase in IRCE-binding activity compared to prior exposure to PPI and then PKC. Although both kinases target Ser/Thr amino acids, it is likely that the sites of Sp1 phosphorylation are different for each kinase. That treatment with PPI and kinase is not simply an addition of the activities of each enzyme points to the idea that these proteins target selected Sp1 site(s), which are specific for each kinase and PPI. Furthermore, it is tempting to speculate that in order for Sp1 to gain or lose IRCE binding activity, a set order of exposure to either kinase or PPI is needed to yield a specific outcome. The targeting of specific site(s) in Sp1 by the kinases and PPI offers a potential way to fine tune the activity of Sp1 in response to different signaling pathway activities. Future studies will be needed to confirm this possibility.

Although our results show that Sp1 interacts with the apo AI IRCE and this interaction mediates the stimulatory actions of insulin, the finding is not novel because previous studies showed that Sp1 also binds to other insulin response element-(s) present in the fatty acid synthase, FAS gene (*38*). However, in contrast to the apparent simplicity of the Sp1 interaction with the apo AI IRCE, its interaction with the insulin responsive motif(s) in the FAS gene is more complex. The insulin induction of the apo AI gene appeared to be solely mediated by the IRCE but there are several such elements in the FAS gene (*39*). The FIRE2, FIRE3, hFIRE, and ICE cis-acting elements are all thought to participate in the FAS response to insulin. These elements may interact with a variety of factors and the response is dependent on cell model. For example, FIRE2 is believed to bind USF-1 and -2, but the hFIRE (−57 to −34) binds both Sp1 and Sp3. Sp1 enhances but Sp3 blocks the activity of hFIRE. Like FAS, Sp1 enhances activity of the apo AI IRCE, but overexpression of Sp3 had no effect on activity of this motif (Figure 2). Furthermore, at least two of the insulin responsive motifs in the FAS gene are positioned adjacent to a NF-Y element, and the binding of this factor also influences the actions of the hormone responsive motifs (*39*). In addition to FAS, leptin gene transcription under the control of the −101 to −83 sequences also requires Sp1 for induction by insulin (*40*). This comparative analysis suggests that the interaction between Sp1 and the IRCE has some similarities but also differences from other insulin responsive motifs in FAS and leptin. The main point is that Sp1 appears to mediate insulin stimulation of several genes.

It is likely that Sp1 is not the only factor that mediates the actions of insulin. Other transcription factors have been found to mediate expression of other insulin responsive genes. For example, the GAPDH gene is also enhanced by the actions of insulin and this induction requires the insulin responsive element, IRE. The IRE is comprised of at least two regions, A and B. IRE-A is homologous to the apo AI IRCE, but despite the sequence similarity, the motif binds to a nuclear protein called IRE-ABP (*11*). The amino acid sequence of IRE-ABP shows that it belongs to the HMG-class of proteins and shares common features with SRY and TCF-1, both of which may also bind to IRE-A (*12*). The mechanism(s) by which the interaction of IRE-ABP with its recognition site mediates the actions of insulin is not yet clear. But the ability of IGF peptides to activate the IRE-ABP involves phosphorylation of the protein (*12*). Although the IRCE and IRE-A share sequence similarities, the actions of insulin may require nuclear factors other than Sp1. We have restricted our comparisons of the apo AI IRCE with motifs that mediate the positive actions of insulin and not to genes that are inhibited by the hormone.

In summary, we have studied insulin regulation of apo AI as a model of hormonal induction of this gene. Insulin, its mimetic, bpv, and PDBu stimulate apo AI gene transcription, and the actions of these agents are mediated by a single cis-acting element, the IRCE, a motif that binds Sp1. The signaling pathways triggered by each agent require the participation of Sp1 and appears to involve phosphorylation of Sp1. The terminal kinase, MAPK or PKC, at the end of the signaling cascades activated by the agents alters the IRCE binding activity of Sp1. An increase or decrease in the binding of Sp1 to the IRCE appears on the surface to be dependent on the kinase or PPI and the order of exposure to these enzymes.

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