Insulin Stimulates and Diabetes Inhibits O-Linked *N*-Acetylglucosamine Transferase and O-Glycosylation of Sp1

Gipsy Majumdar,[1] Jeremiah Wright,[2,3] Paul Markowitz,[1] Antonio Martinez-Hernandez,[1,4,5] Rajendra Raghow,[1,6] and Solomon S. Solomon[1,2,6,7]

Insulin stimulates both the biosynthesis of transcription factor Sp1 and its O-linked *N*-acetylglucosaminylation (O-GlcNAcylation), which promotes nuclear localization of Sp1 and its ability to transactivate calmodulin (CaM) gene transcription. To investigate this further, we incubated H-411E liver cells with insulin (10,000 μU/ml) and quantified the subcellular distribution of O-GlcNAc transferase (OGT) and O-GlcNAc–modified Sp1. We also examined the phosphorylation of Sp1 using both Western blot and incorporation of ^{32}P into Sp1. The results demonstrate that insulin, but not glucagon, stimulates OGT synthesis and enhances cytosolic staining of OGT (histochemical). Insulin increases O-GlcNAc-Sp1, which peaks at 30 min, followed by decline at 4 h. In contrast, insulin initiates phosphorylation of Sp1 early, followed by a continued increase in phosphorylated Sp1 (PO_4-Sp1) at 4 h. A reciprocal relationship between O-GlcNAc-Sp1 and PO_4-Sp1 was observed. To explore the pathophysiological relevance, we localized OGT in liver sections from streptozotocin (STZ)-induced diabetic rats. We observed that staining of OGT in STZ-induced diabetic rat liver is clearly diminished, but it was substantially restored after 6 days of insulin treatment. We conclude that insulin stimulates CaM gene transcription via a dynamic interplay between O-glycosylation and phosphorylation of Sp1 that modulates stability, mobility, subcellular compartmentalization, and activity. *Diabetes* 53:3184–3192, 2004

In addition to its well known effects on the plasma membrane, insulin regulates nuclear events (1,2). Binding of insulin to its receptor triggers a cascade of intracellular signals, leading to posttranslational modification of numerous proteins that include transcription factors capable of selectively reprogramming gene expression. Insulin briskly enhances transcription of the calmodulin (CaM) gene, and we have shown that both basal and insulin-stimulated transcription of CaM is critically dependent on transcription factor Sp1 (3,4). Furthermore, insulin positively regulates the steady-state levels of Sp1 (5) as well as its posttranslational modifications that precede CaM gene transcription (6). The two major types of insulin-mediated posttranslational modifications of Sp1 are O-glycosylation and phosphorylation. O-glycosylation is a modification that involves covalent linkage of the monosaccharide *N*-acetylglucosamine (GlcNAc) to serine and threonine residues of the protein (7). O-glycosylation occurs dynamically on many nuclear and cytoplasmic proteins, and most of the O-linked GlcNAcylated (O-GlcNAcylated) proteins can also be phosphorylated (8,9). Furthermore, O-GlcNAc is reciprocal with phosphorylation on some well-studied proteins and may be involved in regulating biological functions in eukaryoctes (10,11). We have demonstrated that O-glycosylation regulates the intracellular compartmentalization, stability, and activity of Sp1 (6,7,12). O-GlcNAcylation of Sp1 is modulated in response to changing levels of intracellular glucose (11). It has been suggested that a balance between O-glycosylation and phosphorylation is a crucial determinant of both the activity of Sp1 and its ability to regulate target genes. The dynamics of O-linked glycosylation and phosphorylation of Sp1 and how the two processes may be regulated by insulin are not well defined. During O-glycosylation, the enzyme O-GlcNAc transferase (OGT) uses the substrate uridine-5′-diphosphate (UDP)-GlcNAc to attach a single GlcNAc moiety to the serine and threonine residues of a target protein (9,10). It now appears that OGT plays an important role in both O-glycosylation and transcription (13).

Our studies present details of these alterations and interactions, including a critical role for OGT in insulin action. In addition, we demonstrate that a loss of OGT protein occurs in the livers of insulin-deficient rats and is restored by insulin treatment. This effect potentially places OGT at a central position in the pathophysiology of type 1 diabetes and diabetic ketoacidosis. We also show that a reciprocal relationship may exist between glycosylated Sp1 and phosphorylated Sp1. Thus, insulin stimulates OGT, which O-glycosylates the insulin-enhanced levels of Sp1. The O-GlcNAc-Sp1, which is more stable, rapidly migrates to the nucleus, where it is partially or wholly

From [1]Research Services, VA Medical Center, Memphis, Tennessee; [2]Medical Services, Veterans Affairs Medical Center, Memphis, Tennessee; the [3]Health Science Center, University of Tennessee, Memphis, Tennessee; [4]Pathology Service, Veterans Affairs Medical Center, Memphis, Tennessee; the [5]Department of Pathology, Health Science Center, University of Tennessee, Memphis, Tennessee; the [6]Department of Pharmacology, Health Science Center, University of Tennessee, Memphis, Tennessee; and the [7]Department of Medicine, Health Science Center, University of Tennessee, Memphis, Tennessee.

Address correspondence and reprint requests to Sol Solomon, MD, Research Service (151), 1030 Jefferson Ave., Memphis, TN 38104. E-mail: ssolomon@utmem.edu.

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DAB, 3,3′-diaminobenzidine tetrahydrochloride; GlcNAc, *N*-acetylglucosamine; HRP, horseradish peroxidase; O-GlcNAc, O-linked GlcNAc; OGT, O-GlcNAc transferase; STZ, streptozotocin; UDP, uridine-5′-diphosphate.

deglycosylated and then phosphorylated. These carefully orchestrated interactions regulate CaM gene expression.

RESEARCH DESIGN AND METHODS

Insulin and protein standards were obtained from Sigma (St. Louis, MO). Nuclear isolation kit, deoxynorleucine, and streptozotocin (STZ) were also purchased from Sigma. Protease inhibitors were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Rabbit polyclonal anti-Sp1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti–O-GlcNAc antibody was obtained from Affinity BioReagents (Golden, CO). Anti–OGT antibody was a kind gift of Dr. G. Hart, Department of Biochemistry, John Hopkins University Medical School, Baltimore, MD. Protein A Sepharose, protein assay reagent, and kaleidoscope protein molecular weight markers were obtained from Bio-Rad (Hercules, CA). [^{32}P]orthophosphate was purchased from Perkin Elmer (Boston, MA).

All procedures were performed in accordance with Public Health Services (National Institutes of Health) guidelines for the care and use of animals and were approved by the institutional animal care and use committees at the University of Tennessee and Veterans Affairs Medical Center (AAALAC [Association for Assessment and Accreditation of Laboratory Animal Care]-accredited facilities). Male Holtzman rats were rendered diabetic by injection of 65 mg/kg STZ via the tail vein (14,15). After injection the rats were maintained on chow (Ralston Purina, St. Louis, MO) in metabolic cages, with daily measurements of weight, urine volume, glucose, and ketones. At the time of death, blood glucose and plasma insulin were measured (5). Untreated STZ-induced diabetic rats were killed on day 3, insulin-treated STZ-induced diabetic rats (6 units 70N/30R insulin per day) on day 5, and control rats (saline) on day 6. The clinical parameters of diabetes were assessed in the different groups of rats and has already been previously reported (5).

Cell culture. Minimal deviant rat hepatoma H411E cells were obtained from ATCC (American *Type Culture* Collection) and were grown in Eagle's minimum essential media supplemented with 1% glutamine, 1% nonessential amino acids, 1% streptomycin/penicillin, and serum (10% calf serum and 5% fetal bovine serum). Cells were cultured at 37°C in 5% CO_2 and 95% air in a humidified incubator (6).

Experimental procedure for immunoblotting. Cells were cultured in 60 × 15-mm diameter sterile petri dishes until 70–80% confluency. Before treatment with either insulin or glucagon, complete growth medium was changed to serum-free medium (Eagle's minimum essential media, 1% glutamine, nonessential amino acids, and antibiotics) for 36–40 h. Triplicate or duplicate cell cultures were treated as outlined for each experiment, and all experiments were repeated at least three times. After 4 h of hormone treatment, total protein was extracted from the cells, as described previously (12). Briefly, cells were washed twice with phosphate buffer and radioimmunoprecipitation assay buffer (1 × phosphate buffer, 1% igepal CA-230 [Sigma], 0.5% sodium deoxycholate, 0.1% SDS) containing 0.5 mmol/l phenylmethylsulfonyl fluoride, 0.5 mmol/l dithiothreitol, 1.0 mmol/l sodium orthovanadate, aprotinin, and protease inhibitor cocktail. Cells were scraped, collected into an Eppendorf tube, and then passed through a 21-G needle to disrupt them. Homogenized cells were kept on ice for 30–60 min and then centrifuged at 10,000*g* for 10 min. The supernatant was collected and the protein content quantitated, using the Bio-Rad protein assay (12).

For the immunoprecipitation reaction, 500 μg of protein was added to 4 μg of anti-Sp1 antibody in binding buffer (10 mmol/l Tris HCl, pH 7.9, 2 mmol/l $MgCl_2$, 0.15 mmol/l NaCl, 1 mmol/l dithiothreitol, 10% glycerol, and 1 mmol/l phenylmethylsulfonyl fluoride) at a final concentration of 1 μg protein/μl and incubated at 4°C overnight. Protein A Sepharose (20 μl) was then added, and the mixture was incubated at 4°C on a rocker platform for 2 h. The antibody–protein A complexes were pelleted by centrifugation (1,000*g*) and then washed four times with binding buffer. The pellets were resuspended in 1 × SDS sample buffer, boiled, and analyzed by SDS-PAGE.

For Western blot analysis, equal amounts of protein from each sample were separated using 7.5% SDS-PAGE. After electrophoresis, the protein samples were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA) using a Trans-Blot electrophoretic transfer cell (Bio-Rad). Western-blot analyses were conducted using either rabbit polyclonal anti-Sp1 antibody (1:5,000) or monoclonal anti–O-GlcNAc antibody (1:1,000) followed by horseradish peroxidase (HRP)-conjugated second antibody. To quantify the protein, a chemiluminescent signal was developed using detection reagents from the ECL Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ), and the signal was recorded on X-ray film. The blots probed with anti–O-GlcNAc antibody were stripped and reprobed with anti-Sp1 antibody to determine total Sp1. Blots were stripped and probed again with anti-actin antibody (1:10,000) to determine loading and specificity of hormone effect. The data from individual gels for Sp1, O-GlcNAc, and actin were collected and subjected to statistical analysis.

Immunocytochemical staining of OGT. Cells were grown in RS-coated glass chamber slides (Nalge Nunc, Naperville, IL). After achieving 60% confluency, cells were treated overnight (12–16 h) with serum-free medium containing insulin and other agents. In all of these experiments, the cells had already been treated for 12–14 h in serum-free media and then again with hormone overnight (12–16 h). Concentrations of agents used were as follows: insulin 10,000 μU/ml, glucagon 1.5×10^{-5} mol/l. Cells were washed with cold PBS and immediately fixed with 10% neutralized formalin. Immunocytochemical staining was performed with anti-OGT antibody. The signal was developed by HRP-conjugated second antibody, using a 3,3′ diaminobenzidine tetrahydrochloride (DAB)-plus kit from Zymed (San Francisco, CA).

Effects of insulin and glucagon on OGT in rat H411E cells by Western blot analysis. Quiescent, 70–80% confluent H411E cells were treated with either insulin (10,000 μU/ml) or glucagon (1.5×10^{-5} mol/l), and the cell extracts were subjected to SDS-PAGE and immunoblotting as described above. Western blot analyses were conducted using rabbit polyclonal anti-OGT antibody (1:1,000) followed by HRP-conjugated second antibody. Blots were stripped and probed again with anti–β-actin antibody (1:10,000) to determine loading and specificity of hormone effect.

Phosphorylation and glycosylation of Sp1 in response to insulin. Quiescent, 70–80% confluent H411E cells were treated with or without insulin (10,000 μU/ml) at 0 h, 30 min, and 4-h intervals and metabolically labeled in vitro with [^{32}P]orthophosphate (400 μCi/5 ml). After labeling, Sp1 was immunoprecipitated from 500 μg of the [^{32}P]phosphate-labeled H411E cell lysates and was separated by SDS-PAGE. The radiolabeled proteins were analyzed by a phosphorimager (Molecular Dynamics, Sunnyvale, CA), using a phosphorimager screen. In another set of experiments, H411E cells were treated with or without insulin (10,000 μU/ml) at 0 h, 30 min, and 4-h intervals. Sp1 was then immunoprecipitated from 500 μg of the total cell lysates and was subjected to SDS-PAGE. Parallel Western blot analyses were performed using anti-phosphoserine and anti–O-GlcNAc antibodies.

Phosphorylation of Sp1 in the presence of STZ and insulin. H-411E cells were treated with or without insulin in the presence of STZ. After these treatments, 500 μg of total proteins extracted from the cells were subjected to anti-Sp1 antibody immunoprecipitation to assess both O-phosphorylation and total Sp1 by Western blot. Blots were sequentially probed with monoclonal anti–O-phosphoserine antibody, stripped, and reprobed with polyclonal anti-Sp1 antibody.

Statistical analysis. Bands in the X-ray films were scanned and quantified using the Quantity One software program from Bio-Rad, with a Macintosh G-3 computer. Mean, standard deviation, standard error, and Student's *t* tests were calculated using the Excel program. These data were then grouped and analyzed statistically as shown. For paired *t* tests, this is so stated; if unpaired, it is referred to simply as Student's *t* test.

RESULTS

Effects of insulin and glucagon on OGT in rat H411E cells by Western blot analysis. Data from previous work (6) have demonstrated insulin's powerful effect on stimulating both Sp1 synthesis and its O-glycosylation. These events precede markedly enhanced transcription of the CaM gene (5,6). To determine whether O-glycosylation of Sp1 by insulin was initiated by activation of OGT, we incubated H411E liver cells with either insulin or glucagon and assessed OGT levels by Western blotting. A representative Western blot (Fig. 1*A*) shows that insulin but not glucagon clearly stimulates the two OGT-specific 110-kDa and 78-kDa bands. The quantification from multiple experiments in Fig. 1*B* and *C* demonstrates that whereas insulin significantly increased both 110-kDa ($P < 0.03$) and 78-kDa ($P < 0.02$) OGT-specific polypeptides, glucagon did not stimulate either band (P = NS). Insulin stimulation of the 110-kDa band is clearly more intense (+50%) than the 78-kDa band (+20%).

Effects of insulin and glucagon on OGT in cultured rat hepatoma (H411E) cells by immunohistochemistry. To further define the effects of these hormones on intracellular levels of OGT, we studied the localization of this enzyme by immunohistochemical staining, using spe-

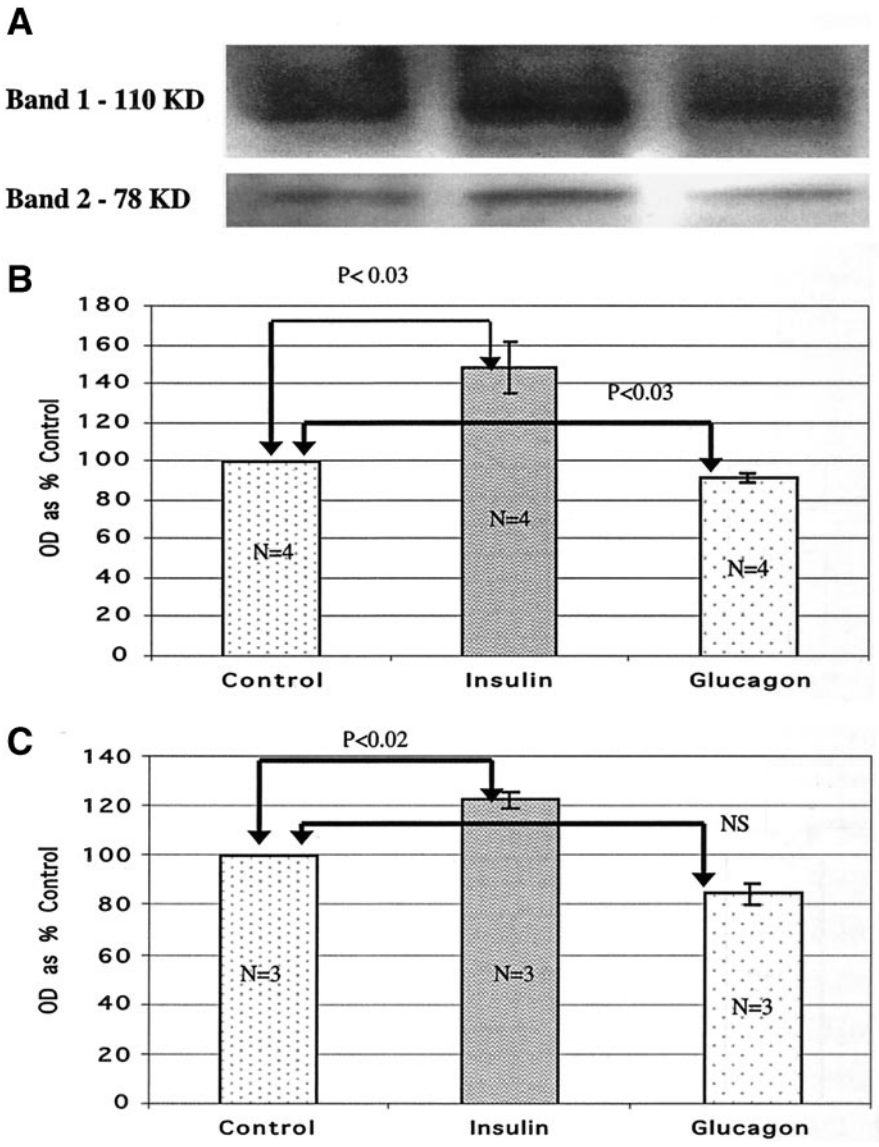

FIG. 1. Effects of insulin and glucagon on OGT extracted from H411E cells. Cells were incubated with either insulin (10,000 μU) or glucagon (1.5×10^{-5} mol/l) for 4 h. *A*: Equal amounts of cell extracts were immunoblotted and probed with antibodies to OGT, and then the membrane was stripped and reprobed for β-actin. β-Actin values did not change significantly with these treatments (data not shown). *B* and *C*: Statistical presentation of normalized optical density (OD) values of Western immunoblots of 110-kD protein (*band 1*) (*A*) and 78-kD protein (*band 2*) (*B*) from multiple experiments. Values are shown as the means ± SE. Statistical significance is as indicated in the individual figures.

cific antibodies (Fig. 2). Control cells exposed to neither of the hormones had only faint equivocal reactivity with the antibodies, showing a predominantly blue background stain (Fig. 2*A*). Cultured cells treated with glucagon (Fig. 2*B*) had weak reactivity, as demonstrated by minimal brown staining. In contrast, cells treated with insulin (Fig. 2*C*) had an intense, diffuse cytoplasmic brown reaction product. There was no demonstrable reaction product in the nuclei, although it is possible that some HRP reactivity could have been obscured by the hematoxylin stain. This can be seen even more clearly at higher magnification (Fig. 2*D*). Furthermore, cells simultaneously exposed to insulin and glucagon had considerably less demonstrable OGT antigen than cells exposed to insulin alone, but they clearly had more than control cells (data not shown). Thus, the intracellular levels of OGT are regulated by glucagon and insulin in an opposite manner, consistent with the well-known physiological antagonism between these two hormones.

Localization of OGT in diabetic rat liver with or without insulin treatment. Because most of our observations were made in H 411E liver cells, we extended these studies to the liver in STZ-induced diabetic rats. Clinical data from the STZ-induced diabetic rats on the 3rd day after treatment showed that they lost 27 g of body weight, had a plasma glucose of 436 mg% (control 95 mg%), had a plasma insulin of 0.3 ng/ml (control 10 ng/ml), and had urine volume of 84 ml/24 h (control 12 ml/24 h.) with positive glucose and ketones. The insulin-treated STZ-induced diabetic group lost only 16 g of body weight in 6 days, had plasma glucose of 60 mg%, and had plasma insulin of 84 ng/ml (insulin treated); however, they still demonstrated increased urine volume, but with less urinary glucose and ketones than untreated STZ-induced diabetic rats (5,14,15).

Data from diabetic and insulin-treated STZ-induced diabetic animals are shown in Fig. 3. Livers were removed from animals after death and fixed in formalin. Later, sections were cut from these livers and stained with anti-OGT antibody and a second antibody tagged with HRP. Normal rat liver contained abundant OGT antigen in hepatocytes and bile duct cells (Fig. 3*A* and *D*). Hepatocytes in perivascular locations (periportal and pericentral) had slightly more intense staining than mid-lobular hepatocytes (Fig. 3*A* and *G*). OGT was most easily identified in the cytoplasm, and at high magnification, cytoplasmic

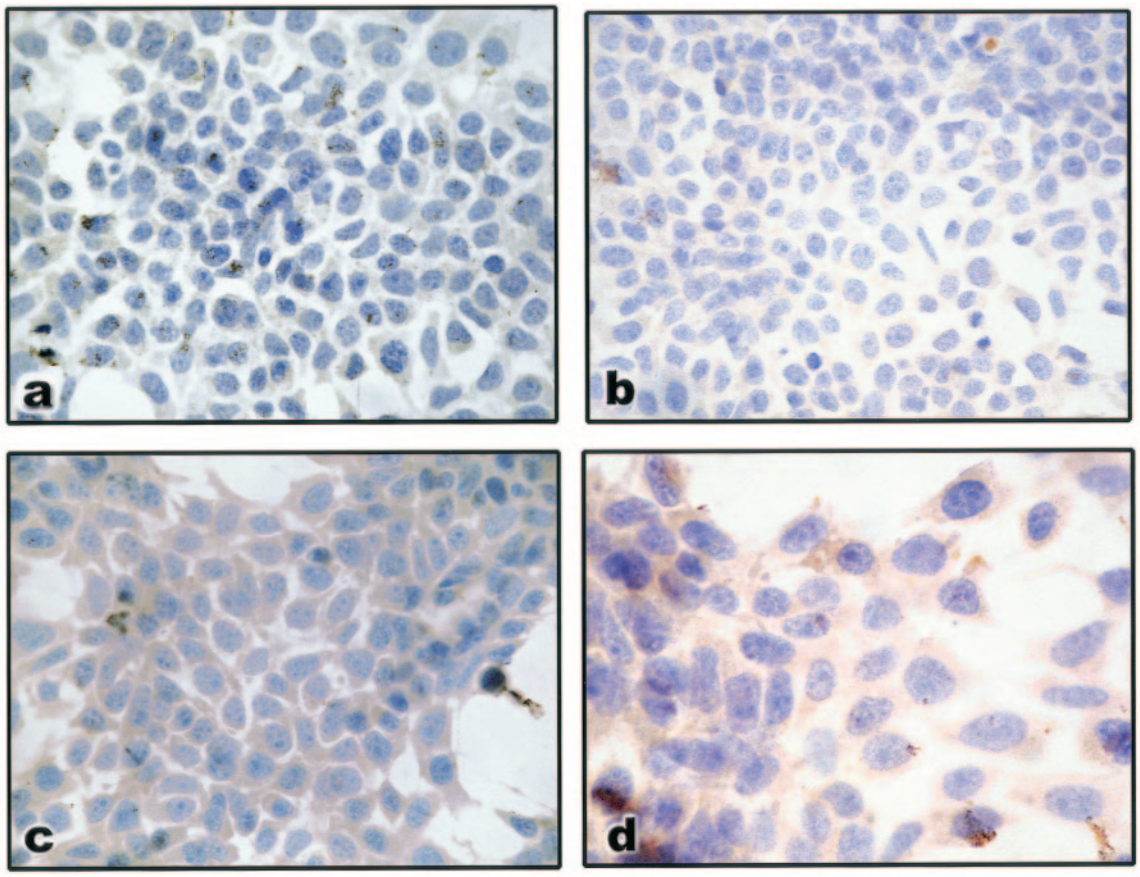

FIG. 2. Immunohistochemical localization of OGT in cultured hepatoma cells. All sections reacted with HRP-DAB system (see RESEARCH DESIGN AND METHODS). The reaction product demonstrating the localization of the antigen is brown. Sections counterstained with hematoxylin are blue. *a*: Cultured cells without any hormonal treatment (control cells). The cytoplasm contains barely detectable levels of OGT. *b*: Glucagon-treated cells. Compared with control cells (*a*), there is minimally increased cytoplasmic staining. *c*: Insulin-treated cells. All cells contain clearly demonstrable OGT. *d*: Insulin-treated cells with higher magnification, demonstrating the diffuse cytoplasmic staining. Some nuclear reactivity could be obscured by the hematoxylin stain. Magnification ×400 (*a*, *b*, and *c*) and ×600 (*d*).

OGT staining had a distinct granular appearance (Fig. 3*G*), suggesting the OGT is confined to some subcellular compartment. Again, the hematoxylin counterstain may be obscuring some OGT staining in the nucleus. Interestingly, most mesenchymal cells in the liver (endothelial, Kupfer, smooth muscle, and Ito cells) lacked demonstrable OGT-specific staining; only a few interstitial cells in the portal spaces had some occasional reactivity (Fig. 3*D*). The liver of diabetic rats without insulin treatment had a dramatic reduction in the content of demonstrable OGT antigen in the hepatocytes (Fig. 3*B*, *E*, and *H*). Here, most hepatocytes showed no staining; only a few scattered cells and particularly perivascular hepatocytes had demonstrable OGT antigen (Fig. 3*B*, *E*, and *H*). There was no significant change in the reactivity of mesenchymal cells (Fig. 3*H*). The livers of diabetic rats treated with insulin had a pattern and intensity of OGT-specific staining similar to that of liver from normal nondiabetic rats (Fig. 3*C*, *F*, and *I*).

Relationship between phosphorylation and glycosylation of Sp1 in H411E cells in the presence of insulin. To assess the potential interplay between O-glycosylation and O-phosphorylation of Sp1, we stimulated H411E cells with insulin at different time intervals, as indicated, to assess the relationship between glycosylation and phosphorylation. Data shown in Fig. 4 illustrate the relationship of phosphorylated Sp1 to O-GlcNAc-Sp1 in H411E cells during insulin stimulation. A time study of insulin-induced O-glycosylation of Sp1 by Western blot (average $n = 4$) indicates that insulin stimulated O-GlcNAc-Sp1 production as early as 15 min (data not shown); the steady-state levels of O-GlcNAc-Sp1 peaked at 30 min ($P < 0.01$) and then started to decline (Fig. 4*C*). In contrast, when these samples were probed with an anti-phosphoserine antibody (Fig. 4*B*), a progressive increase in phosphorylated Sp1 was seen over 4 h with insulin. Although the difference in phosphorylated Sp1 production in insulin-stimulated cells was not statistically significant when compared with its controls at 30 min, it became so by 4 h ($P < 0.04$). Figure 4*A* demonstrates ^{32}P-incorporation into Sp1 (which increases rapidly) clearly increased for insulin at 4 h. The experiment shown here is for $n = 1$. An additional experiment was performed that was virtually identical (data not shown). Overall, the data demonstrate an early (30 min) increase in O-GlcNAc-Sp1, decreasing by 4 h, with a concomitant progressive increase in phosphorylated Sp1 over the same time period, best interpreted as reflecting a reciprocal relationship.

Effects of STZ on O-phosphorylation of Sp1 in the presence of insulin. The serine site(s) on Sp1 is found to be involved in O-GlcNAcation and phosphorylation. O-GlcNAcase is the enzyme that removes O-GlcNAc from its protein site. To delineate the reciprocal relationship of

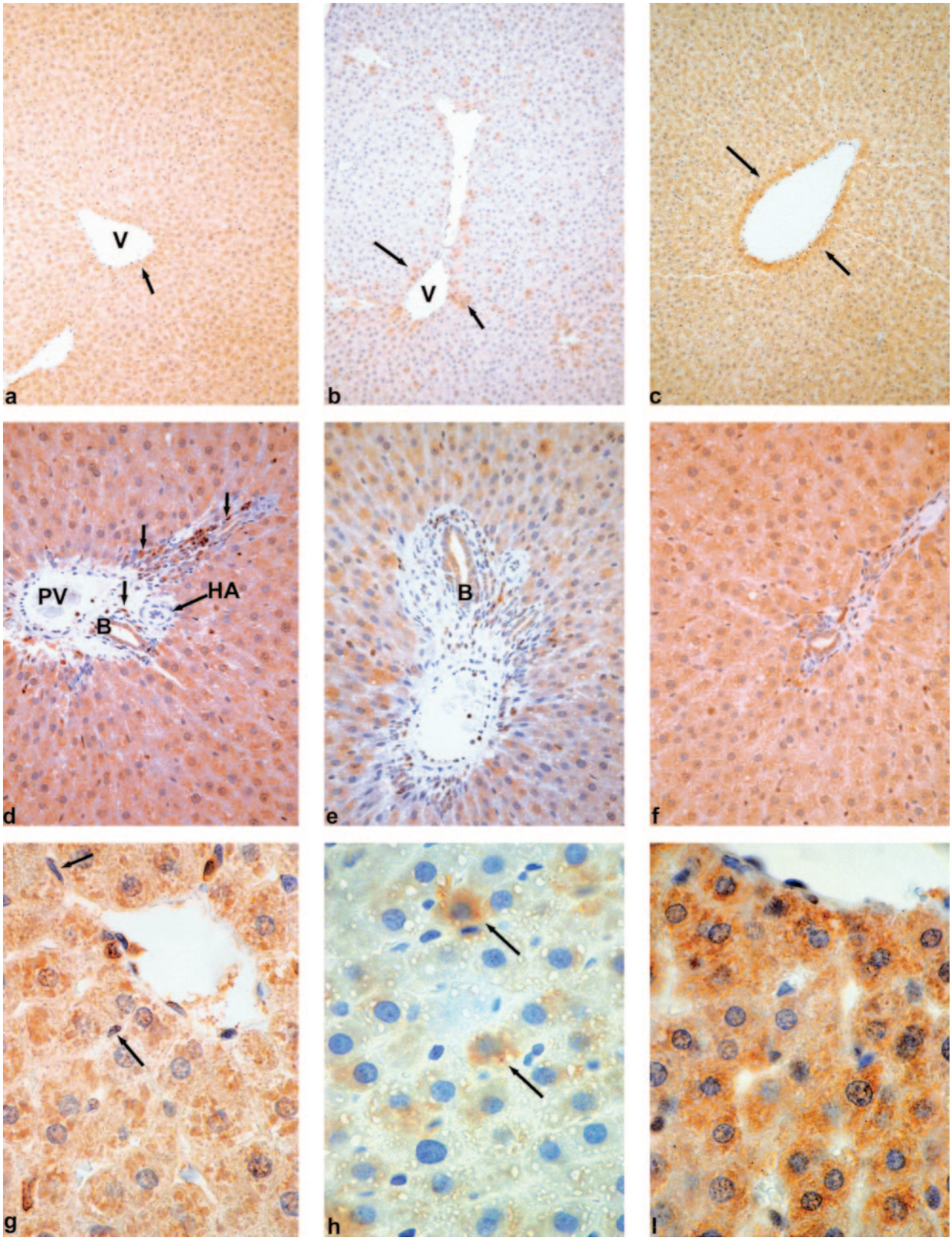

FIG. 3. Immunohistochemical localization of OGT antigen in rat liver. All sections reacted with HRP-DAB system (see RESEARCH DESIGN AND METHODS). The reaction product demonstrating the localization of the antigen is brown. Sections counterstained with hematoxylin are blue. *a*: Normal rat liver. All hepatocytes contain OGT. The hepatocytes surrounding a central vein (V) have a slightly more intense reactivity (arrow). *b*: Untreated diabetic rat liver. There is a dramatic loss of reactivity compared with the normal liver. Most hepatocytes have no demonstrable OGT antigen. Only a few lobular hepatocytes and the perivenular (V) ones (arrows) contain some demonstrable antigen. *c*: Insulin-treated diabetic rat liver. All hepatocytes are staining with an intensity similar to that of normal rat hepatocytes. The preferential staining of perivenular hepatocytes (arrows) is clearly demonstrated. Magnification for *a*, *b*, and *c* was ×100. *d*: Normal rat liver; detail of portal area. All hepatocytes have a positive

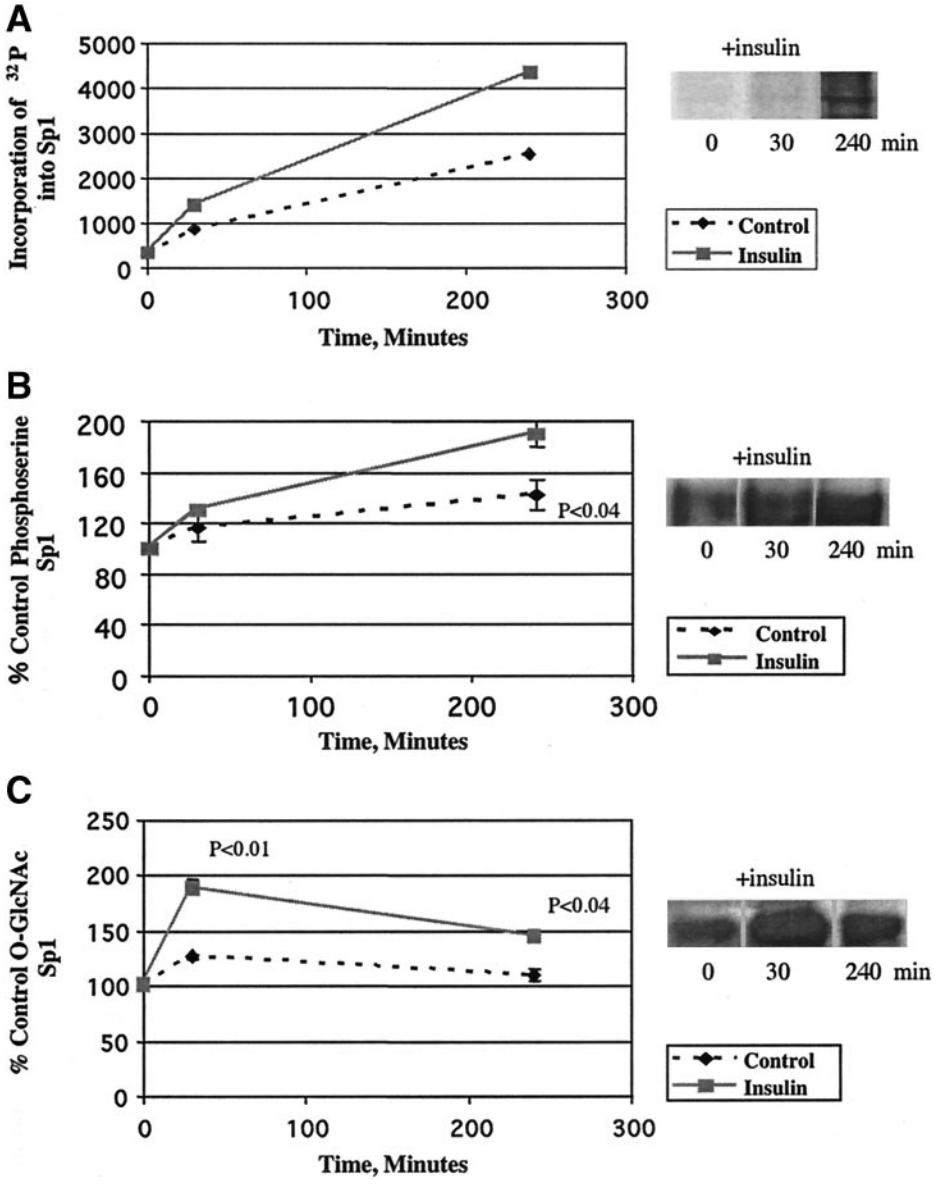

FIG. 4. Effect of insulin on phosphorylation and O-glycosylation of Sp1. *A*: Effect of insulin on ^{32}P incorporation into Sp1 ($n = 1$). Quiescent H411E cells were treated with or without insulin (10,000 μU/ml) at 0 h, 30 min, and 4-h intervals and labeled in vitro with [^{32}P]orthophosphate (400 $\mu Ci^{-1} \cdot$ petri dish$^{-1} \cdot 5$ ml^{-1}). Sp1 was then immunoprecipitated with anti-Sp1 antibody from 500 μg of the [^{32}P]phosphate-labeled H411E cell lysates and separated by SDS-PAGE. The radiolabeled protein was analyzed by Phosphorimager (Molecular Dynamics) using a phosphorimager screen. Densitometrically analyzed values of immunoblots of phosphorylated Sp1 are shown. In another set of experiments, quiescent H411E cells were treated with or without insulin (10,000 μU/ml) at 0 h, 30 min, and 4-h intervals, and the cell lysates were extracted for protein. The immunoprecipitated Sp1 from the cell lysates was subjected to SDS-PAGE as detailed above. *B*: Effect of insulin on serine phosphorylation of Sp1 ($n = 3$). Western blot analysis was performed using anti-phosphoserine antibody, and densitometrically analyzed values of the blots normalized from multiple experiments are presented. *C*: Effect of insulin on O-glycosylation of Sp1 ($n = 4$). Western blot analysis was performed using anti–O-GlcNAc antibody, and densitometrically analyzed values of the blots normalized from multiple experiments are presented. Values are shown as the means ± SE. Statistical significance is as indicated in the individual figures. In the upper right-hand corner of each panel (*A*, *B*, and *C*), a representative phosphorimager blot (*A*) or Western blot (*B* and *C*) is presented.

O-GlcNAcylation to phosphorylation on the critical serine sites after insulin treatment, we used STZ, which is an inhibitor of O-GlcNAcase, in the presence of insulin to produce hyperglycosylated Sp1 and then quantitated the fraction of phosphorylated Sp1 under these conditions. The immunoprecipitated Sp1 was subjected to SDS PAGE and probed with anti-phosphoserine antibody to quantitate the amount of phosphorylated Sp1. Figure 5*A* shows a typical Western blot, and quantitation of these data from three experiments is shown in Fig. 5*B*. Insulin clearly stimulated phosphorylation of Sp1, as did STZ alone. However, in the presence of STZ and insulin, a reduction in O-PO_4-Sp1 ($P < 0.001$) was observed (see DISCUSSION).

DISCUSSION

We have previously established a critical role of Sp1 transcription factor protein as a mechanism for stimulating CaM gene transcription in response to insulin (3,4). In the absence of insulin, Sp1 levels are reduced and then restored to normal with insulin treatment both in the livers of STZ-induced diabetic rats and in H411E liver cells in culture (5). We also demonstrated that insulin-mediated enhanced levels of total and O-glycosylated Sp1 and its nuclear localization are intimately involved in CaM gene transcription (6). Although glucagon, which generally antagonizes insulin, promoted net Sp1 synthesis, it neither

reaction. The bile ductule cells (B) are also intensely positive. Endothelial cells lining the portal vein (PV), hepatic artery (HA) radicles, and vascular smooth muscle cells are negative. A few interstitial cells in the portal space (arrows) are positive. *e*: Untreated diabetic rat liver; detail of portal area. Only a few hepatocytes have some positivity. Bile ductule cells (B) and even the interstitial cells have notable weaker staining. *f*: Insulin-treated diabetic rat liver; detail of portal area. The staining of all cell types, hepatocytes, ductular, and interstitial cells is similar to that in the normal rat liver. Magnification for *d*, *e*, and *f* was ×200. *g*: Normal rat liver, detail of central region. Hepatocytes have an intensely positive reaction. The staining is not homogeneous throughout the cytoplasm, but clearly granular, suggesting OGT is confined in some subcellular compartment. Sinusoidal lining cells (either Kupfer or endothelial cells) are negative (arrows). *h*: Diabetic rat liver, detail of central region. Only two hepatocytes (arrows) in this field are positive. At this magnification the multiple intracellular lipid vesicles (steatosis) common in uncontrolled diabetes are obvious. All nonparenchymal cells are negative. *i*: Insulin-treated diabetic rat, detail of central region. All hepatocytes have the same intense, granular staining. There is no steatosis. Nonparenchymal cells are negative. Magnification for *g*, *h*, and *i* was ×400.

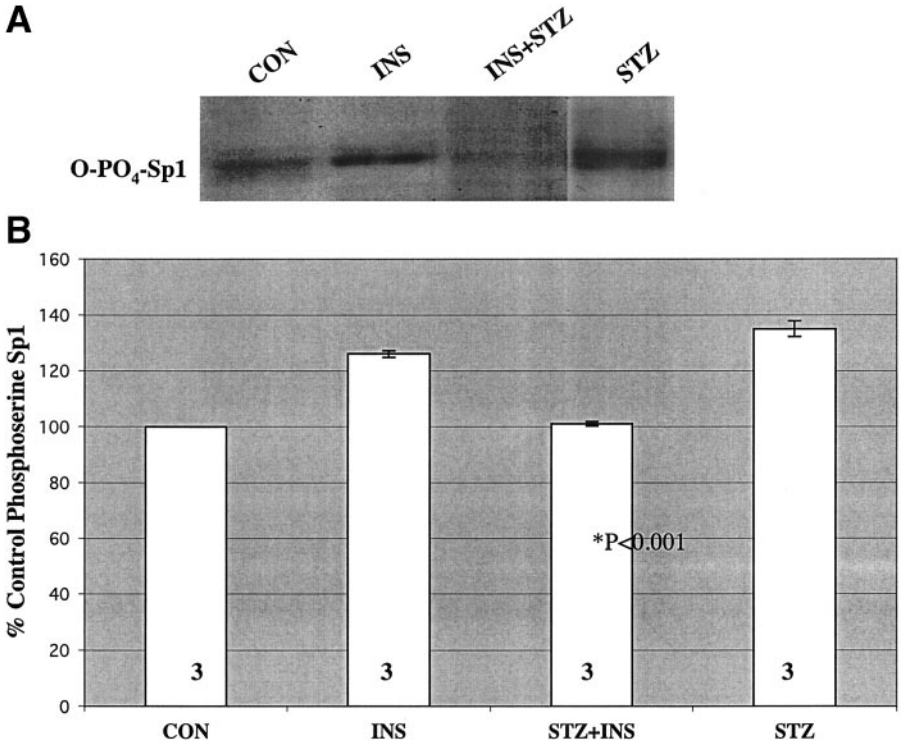

FIG. 5. Effect of STZ on phosphorylation of Sp1 in the presence of insulin. H411E cells were treated with or without insulin (10,000 μU/ml) in the presence of STZ (5 mmol/l) as described. As detailed in RESEARCH DESIGN AND METHODS, the immunoprecipitated Sp1 was subjected to SDS-PAGE, and Western blot analysis was performed using anti-phosphoserine antibody. *A*: Densitometrically analyzed values of the blots normalized from multiple experiments are presented. *B*: Quantitation of the data in *A* from three experiments.

enhanced Sp1's O-glycosylation nor its movement to the nucleus, and hence glucagon did not enhance CaM gene transcription (12). This highly dynamic glycosylation/deglycosylation of protein is catalyzed by the enzymes OGT and O-GlcNAcase. We demonstrate here that insulin but not glucagon stimulates OGT, glycosylating Sp1. STZ selectively destroys pancreatic β-cells and induces an insulin-deficient type 1 diabetic rat model. STZ-induced diabetic rats have low levels of OGT in their liver, and OGT levels are restored to normal values when treated with insulin. We also show that insulin stimulates both O-glycosylation and phosphorylation of Sp1, but by different kinetics. The relationship of O-glycosylation to phosphorylation of Sp1 appears to be reciprocal, i.e., when O-glycosylation levels decrease, phosphorylation levels increase.

OGT, catalyzes the attachment of O-GlcNAc moeties to the serine and threonine residues of intracellular proteins and plays an important role in the hexosamine pathway (16). The end product of the hexosamine pathway, UDP-GlcNAc, is utilized for the glycosylation process by OGT. OGT has been detected in a wide range of tissues including rat liver (16), pancreatic β-cells, and brain cells (17,18). As would be expected, there are abundant O-GlcNAc–modified proteins in these tissues (17,18). It has also been suggested that OGT may participate in glucose sensing and/or the regulation of insulin secretion (19). Our data indicate that detectable levels of OGT are present in normal rat liver and in H411E cells that contain 110-kDa and 78-kDa OGT proteins, as shown by Akimoto et al. (18). Herein, we demonstrate that STZ-induced diabetic animals have low OGT levels that are restored to normal after insulin treatment. In the H411E cells, insulin, but not glucagon, significantly stimulated OGT levels. The O-GlcNAc–modified Sp1 is resistant to proteosomal degradation (20).

O-GlcNAc is a modification of serine and threonine residues of nuclear and cytoplasmic proteins by O-linked β-GlcNAc. Most of the known O-GlcNAcylated proteins are also able to be phosphorylated, generating phosphoproteins that form reversible multimeric protein complexes (9,10). Because O-GlcNAc and O-phosphate may be attached to a serine or threonine on the same or closely related sites (9), it has been proposed that a reciprocal relationship between O-GlcNAcylation and O-phosphorylation may be involved in regulating biological functions in eukaryotes (10,13).

O-GlcNAc and O-phosphate have been detected on many other transcription factors (8). The ubiquitous transcription factor Sp1 is extensively modified by O-GlcNAc (7) and phosphorylated at multiple serine and threonine residues (21,22), but whether these modifications regulate Sp1 function is controversial. Mice challenged with lipopolysaccharide had dephosphorylated Sp1 with reduced binding activity. However, lipopolysaccharide had no effect on Sp1 glycosylation (21). The dynamic interplay between O-glycosylation and phosphorylation is thought to regulate several nuclear and cytoplasmic proteins (23,24). The c-Myc protein can be both glycosylated and phosphorylated at a specific threonine site (Thr-58), and the interplay between these modifications may modulate subcellular trafficking of the protein. Moreover, it has been proposed that O-GlcNAc/O-phosphate modulates turnover of other proteins (23,25). We show here that insulin stimulates both glycosylation and phosphorylation, but that the kinetics of each reaction are different and distinct. During insulin stimulation, glycosylation of Sp1 increases, peaks at 30 min, and slowly declines at 4 h, whereas insulin initiates phosphorylation of Sp1 as early as 30 min and peaks at 4 h (Fig. 4). Kamemura and Hart (22) recently proposed that Thr-58 glycosylation of c-Myc in HL-60 cells occurs before its phosphorylation. Ye and Liu (21) showed that phosphoserine and phosphothreonine bands became

undetectable at 60 and 120 min postlipopolysaccharide but reappeared at 240 min when Sp1 DNA binding activity was partially restored.

From our data, a reciprocal relationship exists between phosphorylation and glycosylation of Sp1 in H 411E liver cells after stimulation with insulin. To further examine the relationship of O-GlcNAc to O-phosphate on the serine residues of Sp1, we incubated cells with STZ (a stimulator of O-GlcNAcation), insulin, or a combination of STZ plus insulin. We found that insulin alone increased phosphorylated Sp1, but the combination of STZ plus insulin decreased phosphorylated Sp1 significantly. STZ plus insulin together, being a strong stimulator of O-GlcNAcation (insulin by stimulating OGT and STZ by inhibiting O-GlcNAcase), increases glycosylation significantly when compared to either insulin or STZ alone. If STZ inhibits O-GlcNAcase, then GlcNAc will remain bound to individual serine residues. Without an insulin drive, the total amount of O-GlcNAc-Sp1 will be less than in the presence of insulin, so when STZ is present, those insulin-stimulated phosphorylated serine sites will be O-GlcNAced and not available for O-phosphorylation, and hence there is reduced phosphorylated Sp1 (Fig. 4). A similar increase in the level of O-GlcNAc caused by PUGNAc [O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate], a potent inhibitor of O-GlcNAcase, was accompanied by a decrease in phosphorylation in HT29 cells (26).

Based on our published (6) and current data, and the work of others (21,22,27), it seems plausible that insulin stimulates Sp1 biosynthesis and activates OGT, which O-glycosylates the Sp1, causing its mobilization from the endoplasmic reticulum–Golgi to the nucleus, where it binds to the GC-rich sequences of the CaM gene promoter. Some of the O-GlcNAc residues are removed from Sp1 in the nucleus, where Sp1 is phosphorylated. The phosphorylated Sp1 is most active and maximally promotes CaM gene transcription. However, phosphorylated Sp1 is degraded faster and becomes inactive. This is our working hypothesis, and Hart et al.'s (28) data is consistent with this hypothesis. However, data from Kudlow's laboratory (13) suggest that OGT interacts with a histone deacetylase complex by binding to a corepressor, mSin3A, that effectively targets OGT promoters to inactivate transcription factors and RNA polymerase II by O-GlcNAc modification and selective gene silencing (13,29). This results in inhibition, not stimulation, of gene transcription.

We propose that insulin initially promotes Sp1 synthesis and its O-glycosylation on a unique serine site. With time, the O-glycosylated Sp1 is phosphorylated, and powerful regulatory effects on transcription are produced by these alterations. Thus, insulin- and glucose-responsive genes seem to be regulated by a dynamic interplay between glycosylation and phosphorylation of Sp1. This regulation is also readily demonstrated in models of insulin-deficient diabetes or type 1 diabetes.

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