Insulin Dynamically Regulates Calmodulin Gene Expression by Sequential O-Glycosylation and Phosphorylation of Sp1 and Its Subcellular Compartmentalization in Liver Cells*

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O-Glycosylation and phosphorylation of Sp1 are thought to modulate the expression of a number of genes in normal and diabetic state. Sp1 is an obligatory transcription factor for constitutive and insulin-responsive expression of the calmodulin gene (Majumdar, G., Harmon, A., Candelaria, R., Martinez-Hernandez, A., Raghow, R., and Solomon, S. S. (2003) Am. J. Physiol. 285, E584–E591). Here we report the temporal dynamics of accumulation of total, O-GlcNAc-modified, and phosphorylated Sp1 in H-411E hepatoma cells by immunohistochemistry with monospecific antibodies, confocal microscopy, and matrix-assisted laser desorption and ionization-time of flight mass spectrometry. Insulin elicited sequential and reciprocal post-translational modifications of Sp1. The O-glycosylation of Sp1 and its nuclear accumulation induced by insulin peaked early (~30 min), followed by a steady decline of O-GlcNAc-modified Sp1 to negligible levels by 240 min. The accumulation of phosphorylated Sp1 in the nuclei of insulin-treated cells showed an opposite pattern, increasing steadily until reaching a maximum around 240 min after treatment. Analyses of the total, O-GlcNAc-modified, or phosphorylated Sp1 by Western blot and mass spectrometry corroborated the sequential and reciprocal control of post-translational modifications of Sp1 in response to insulin. Treatment of cells with streptozotocin (a potent inhibitor of O-GlcNAcase) led to hyperglycosylation of Sp1 that failed to be significantly phosphorylated. The mass spectrometry data indicated that a number of common serine residues of Sp1 undergo time-dependent, reciprocal O-glycosylation and phosphorylation, paralleling its rapid translocation from cytoplasm to the nucleus. Later, changes in the steady state levels of phosphorylated Sp1 mimicked the enhanced steady state levels of calmodulin mRNA seen after insulin treatment. Thus, O-glycosylation of Sp1 appears to be critical for its localization into the nucleus, where it undergoes obligatory phosphorylation that is needed for Sp1 to activate calmodulin gene expression.

Diabetes mellitus is a disease of absolute (type I diabetes) or relative (type II diabetes) insulin deficiency. Insulin signaling is initiated by its binding to the plasma membrane receptor, triggering appropriate second messengers, which ultimately result in reprogramming of gene expression in the nucleus (1, 2). Insulin-mediated signal transduction leads to post-translational modification of numerous cytoplasmic and nuclear proteins, including transcription factors; O-glycosylation and phosphorylation are the two most prominent post-translational modifications induced by insulin (2, 3). We have demonstrated earlier that the calmodulin (CaM) gene is an important target of insulin action and that the activity of the transcription factor Sp1 is obligatory for both the constitutive and insulin-mediated enhanced transcription of the CaM gene (4, 5). Furthermore, we have shown that insulin regulates Sp1 at least at two different levels; insulin stimulates the biosynthesis of Sp1 and also regulates both its O-glycosylation and phosphorylation (6–8). We have previously reported that insulin stimulates the production of Sp1. Therefore, our data are consistent with the scenario that insulin-mediated O-GlcNAcylation of Sp1 facilitates its migration to the nucleus, where Sp1 is sequentially deglycosylated and then phosphorylated (7, 8). O-GlcNAcylation of a wide variety of cellular proteins, including signaling molecules and transcription factors, is emerging as an important post-translational modification potentially involved in many regulatory mechanisms of eukaryotic cells (8–12). We originally reported that O-glycosylation of Sp1 was essential for argininosuccinate synthetase gene expression in response to glutamine (13). O’Donnell et al. (14) recently demonstrated that the Ogt gene, which encodes the enzyme O-GlcNAc transferase (OGT), is responsible for the formation of O-GlcNAc-Sp1; inactivation of a functional OGT led to a concomitant decline in O-GlcNAc-modified Sp1 and an increased accumulation of phosphorylated Sp1. Based on these and a number of other observations, it has been postulated that a balance between O-glycosylation and phosphorylation may be an important regulatory mechanism for some proteins (9, 10, 12, 15, 16).

With an objective to define the regulatory dynamics of insulin that enhances both the biosynthesis and posttranslational modification of Sp1, we compared temporal changes in the subcellular distribution of O-glycosylated and phosphorylated Sp1. We used fluorochrome-tagged secondary antibodies to label Sp1, phosphorylated serine-, and O-GlcNAc-specific primary antibodies and visualized changes in subcellular distribution of Sp1 in response to insulin by confocal microscopy. The dynamics of total, O-GlcNAc-modified, and phosphorylated Sp1 as elucidated by confocal microscopy were also quantified by Western blot analysis. Finally, we corroborated the insulin-induced sequen-

The abbreviations used are: CaM, calmodulin; MALDI, matrix-assisted laser desorption and ionization; TOF, time-of-flight; MS, mass spectrometry; STZ, streptozotocin; OGT, O-GlcNAc transferase; PBS, phosphate-buffered saline; IGF, insulin-like growth factor.

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temporal and reciprocal changes in the two posttranslational modifications of Sp1 by MALDI-TOF mass spectrometry. We report that these sequential changes in O-GlcNAcylation and phosphorylation of Sp1 in response to insulin are mechanistically related to its ability to stimulate CaM gene transcription.

EXPERIMENTAL PROCEDURES

Chemicals—Insulin, protein standards, nuclear isolation kit, and streptozotocin (STZ) were purchased from Sigma. Protease inhibitors were purchased from Roche Applied Science. Rabbit polyclonal anti-Sp1 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-O-linked GlcNAc antibody was obtained from Affinity BioReagents (Golden, CO). Anti-phosphoserine monoclonal antibody was obtained from Sigma. This antibody is highly specific for phosphorylated serine, since it did not react with free serine, nonphosphorylated serine, phosphorylated tyrosine, phosphorylated threonine, ATP, or AMP, as judged by enzyme-linked immunosorbent assay. The phosphoserine-specific antibody has a high titer against phosphoryserine/bovine serum albumin and we were able to use it routinely at a 1:10,000 dilution for Western blots. The anti-phosphoserine antibody has been used by many investigators, and its specificity is well documented in the literature (17, 18). Protein A-Sepharose, SYPRO, Bio-Rad protein assay reagent, and kaledoscope protein molecular weight markers were obtained from Bio-Rad. Secondary anti-IgG antibodies raised in either rabbit or donkey labeled with Alexa Fluor 488 (green), 594 (red), and 647 (blue) were purchased from Molecular Probes, Inc. (Eugene, OR).

Cell Cultures—H-411E cells, representing a minimal deviant rat hepatoma cell line, were obtained from ATCC and were grown in Eagle’s minimum essential medium supplemented with 1% glutamine, 1% nonessential amino acids, 1% streptomycin/penicillin, and serum (10% calf serum at 37 °C in 5% CO2 and 95% air in a humidified incubator and routinely sub cultured when they became 90–100% confluent.

Immunoprecipitation and Western Blot Analysis—Cells were cultured in 60 × 15-mm sterile Petri dishes until they reached 70–80% confluence. Before treatment with insulin, complete growth medium was changed to serum-free medium (Eagle’s minimum essential medium, 1% glutamine, nonessential amino acids, and antibiotics) for 36–40 h. Following treatment with insulin (10,000 microunits/ml) for various durations as indicated, total protein was extracted from the cells as described previously (19), with minor modifications. To analyze phosphorylation and O-glycosylation of Sp1 in the presence of STZ and insulin, H-411E cells were treated with or without insulin (10,000 microunits/ml) in the presence of STZ (5 mM) for 4 h. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and radioimmune precipitation buffer (1 × PBS, 1% igepal (CA-230; Sigma), 0.5% sodium deoxycholate, 0.1% SDS) containing 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.0 mM sodium orthovanadate, 0.5 mM aprotinin, and protease inhibitor mixture. Cells were scraped, collected into an Eppendorf tube, and then passed through a 21-gauge syringe 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 was quantified using the Bio-Rad protein assay kit.

For the immunoprecipitation reaction, 500 μg of protein were added to 4 μl of anti-Sp1 antibody in the binding buffer (10 mM Tris-HCl, pH 7.9, 2 mM MgCl2, 0.15 mM NaCl, 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride) to a final concentration of 1 μg of 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 centrifuged (1000 × g), and the pellet was washed four times with binding buffer. The pellets were resuspended in 1× Laemmli 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 Corp., Bedford, MA) using a Trans-Blot electrophoresis transfer cell (Bio-Rad). Western blot analyses were conducted using rabbit polyclonal anti-Sp1 antibody (1:5000), monoclonal anti-O-linked GlcNAc antibody (1:1000), and anti-phosphoserine antibody (1:1000) followed by incubation with horseradish peroxidase-conjugated secondary antibody. To quantify the protein, a chemiluminescent signal was developed using detection reagents from the ECL Plus kit (Amer sham Biosciences), and the signal was recorded on x-ray film. The blots probed with either anti-O-GlcNAc antibody or anti-phosphoserine antibody were stripped and reprobed with anti-Sp1 antibody to determine total Sp1. Western blot membranes were stripped and probed again with anti-actin antibody (1:10,000) to determine the equivalency of protein loading and specificity of insulin effect. The data from individual Western blots representing Sp1, O-GlcNAc, phosphoserine, or actin, were quantified by densitometry and subjected to statistical analysis.

Mass Spectrometry—The details of the method used for the analysis of Sp1 by MALDI-TOF MS have been outlined previously (20–22). Briefly, after fractionation of protein(s) by SDS-PAGE, the gel was fixed in 50% methanol and 7% glacial acetic acid for 30 min and stained with SYPRO Ruby Stain (Bio-Rad) overnight at room temperature. The protein bands were visualized under UV light, and excised gel bands of Sp1 were placed in deionized water. The water was then removed, and the gel was dried in a vacuum centrifuge. Once dried, the gel pieces were subjected to trypsin digestion in situ and processed to be spotted on the MALDI plate and characterized by mass spectrometry as previously reported (20–22). To identify serines involved in O-glycosylation and phosphorylation during insulin stimulation by MALDI-TOF MS, H-411E cells were exposed to insulin at 0, 30, and 240 min. Total protein was extracted from the cells and immunoprecipitated with anti-Sp1 antibodies. The immunoprecipitated Sp1 was subjected to SDS-PAGE, and the protein band corresponding to Sp1 (as identified by reaction with Sp1 antibody on a parallel lane) was excised. The putative Sp1 band was digested with trypsin, extracted from the gel, and subjected to MALDI-TOF MS. The peptide mass fingerprint data were analyzed on the EXPASY server (available on the World Wide Web at us.expasy.org) using ALDENT as the tool to identify proteins and their modifications (20–22).

Cell Fixation and Confocal Microscopy—Cells were cultivated on glass slides, washed in PBS two times (5 min each), and fixed in 10% formalin for 10 min. Slides were washed twice in PBS (5 min each), and cells were made permeable in 0.3% Triton X-100 for 15 min. Next, the cells were incubated in 1:1 cold acetone/methanol solution for 10 min at 4 °C and allowed to air-dry. To block nonspecific antibody-binding sites, cells were preincubated in 1% bovine serum albumin in PBST (PBS containing 0.1% Tween 20) for 1 h. To examine glycosylated Sp1, fixed cells in one well of the two-well glass chamber slides were reacted with anti-Sp1 (1:400) rabbit polyclonal antibody (Santa Cruz Biotechnology) and anti-O-GlcNAc (1:250) mouse monoclonal antibody. The other well was stained for phosphorylated Sp1 using anti-Sp1 antibody and anti-phosphoserine (1:300) mouse monoclonal antibody. The cells were incubated for 1 h with primary antibodies diluted in 1% bovine serum.
Insulin Regulates Sp1 by O-Glycosylation/Phosphorylation

RESULTS

Insulin-induced Subcellular Localization of O-Glycosylated and Phosphorylated Sp1: Confocal Microscopy—In order to directly visualize the subcellular distribution of the total, O-glycosylated, and phosphorylated Sp1, H-411E hepatoma cells cultured in glass chamber slides were exposed to insulin for different time intervals. At the denoted times, changes in total Sp1, O-GlcNAc, and phosphoserine were assessed by highly specific primary antibodies followed by their reaction with secondary antibodies labeled with fluorescent tags of different colors. Fig. 1A demonstrates that in response to insulin treatment, total Sp1 (green) increased over a 240-min period. Although there is a detectable

mRNA Analysis—Cells were treated with insulin for different time intervals. Parallel cultures were also treated with insulin in the presence or absence of STZ. Total RNA was extracted at various intervals after insulin treatment, and mRNA analysis was performed by Northern blot using a cDNA probe for CaM described previously (19).

Phosphorylated Sp1: Confocal Microscopy

In response to insulin, there is some detectable cytoplasmic Sp1 (upper panel, d), which rapidly (30 min) accumulates in the nucleus (upper panel, b). The nuclear levels of Sp1 continue to increase at 60 and 240 min (upper panel, c and d, respectively). The nucleoli are devoid of Sp1 regardless of the duration of insulin treatment or the presence of high levels of Sp1 in the nucleus. In contrast to total Sp1, the levels of O-GlcNAc-specific immunoreactivity also rapidly (30 min) accumulate in the nucleus (middle panel, f) but decreases at 60 min (middle panel, g) and even more at 240 min (middle panel, h). The negative nucleolar staining obtained with anti-Sp1 antibodies is not as obvious with anti-O-GlcNAc antibodies. Co-localization of Sp1 and O-GlcNAc residues demonstrates that O-GlcNAc-modified Sp1 initially (30 min) increases followed by a clear, steady decrease at 60 and 240 min (lower panel, j, k, and l, respectively). It also demonstrates that Sp1 is not homogeneously distributed throughout the nucleus, being absent from the nucleolus and from the nuclear periphery (seen as a rim of blue-purple fluorescence), and is most likely also absent in the nuclear membrane (lower panel, j and k). Magnification is × 200. B: insulin steadily enhances the phosphorylation of Sp1 for up to 240 min. H-411E cells were reacted with anti-Sp1 (green) or anti-phosphoserine (red) antibodies prior to their exposure to insulin (0 min) or 30, 60, and 240 min post-insulin treatment. Anti-Sp1 antibodies demonstrate the pattern shown in A. After insulin treatment, there is a steady increase in phosphorylated residues, first prominently seen in the nucleolus (middle panel, f), then in the nucleolus and nucleus (middle panel, g), and finally, in the nucleolus, nucleus, and cytoplasm (middle panel, h). There is a conspicuous absence of Sp1-specific staining in the nucleus (upper panel, b, c, and d). Unlike the levels of O-GlcNAc-modified Sp1 that initially (30 min) increased followed by a steady decrease at 60 and 240 min (Fig. 1A), there was continued accumulation of phosphorylated Sp1 in insulin-treated H-411E cells (lower panel, j, k, and l). Magnification of all other images is × 200.

albumin in PBST. Cells were then washed in PBST five times (15 min each wash), and the fluorochrome-tagged secondary antibodies were incubated for 1 h in the dark at room temperature: anti-rabbit IgG tagged with Alexa 488 (green) (1:300) to detect anti-Sp1, anti-mouse IgG1 tagged with Alexa 594 (red) (1:400) to detect phosphoserine, and anti-mouse IgG1 tagged with Alexa 647 (blue) (1:400) to recognize O-GlcNAc antibody. Following incubation, cells were washed three times in PBST (15 min each wash). Slides were air-dried and mounted with anti-fading reagent mounting medium containing 4’,6-diamidino-2-phenylindole to detect nuclear staining (Molecular Probes anti-fade kit). Images were obtained using a Zeiss inverted laser-scanning microscope LSM 510 with a confocal scan head and a krypton/argon mixed gas laser. To detect phosphorylation and glycosylation of Sp1 in response to insulin, H-411E cells were incubated with or without insulin (10,000 micrograms/ml) at 0-, 30-, and 240-min intervals. In the presence of STZ and insulin, cells were incubated for 4 h. Confocal microscopy was then performed on cells stained with specific combinations of primary and secondary antibodies as described above.

mRNA Analysis—Cells were treated with insulin for different time intervals. Parallel cultures were also treated with insulin in the presence or absence of STZ. Total RNA was extracted at various intervals after insulin treatment, and mRNA analysis was performed by Northern blot using a cDNA probe for CaM described previously (19).

Statistical Analysis—Protein bands, developed by multiple exposure of x-ray films to assure exposure in the linear range, were scanned and quantified using the Quantity One software program from Bio-Rad with a Macintosh G-3 computer. Mean, S.D., S.E., and Student’s t tests were calculated using the Excel program. These data were then grouped and analyzed statistically as shown. For paired t, this is so stated; if unpaired, it is referred to simply as “Student’s t test.”

Insulin-induced Subcellular Localization of O-Glycosylated and Phosphorylated Sp1: Confocal Microscopy

FIGURE 1. A, insulin triggers accumulation of O-GlcNAc-modified Sp1 in the nucleus. H-411E cells were reacted with anti-Sp1 (green) and anti-O-GlcNAc (blue) antibodies prior to treatment (0 min) or 30, 60, and 240 min post-insulin treatment. In response to insulin, there is some detectable cytoplasmic Sp1 (upper panel, d), which rapidly (30 min) accumulates in the nucleus (upper panel, b). The nuclear levels of Sp1 continue to increase at 60 and 240 min (upper panel, c and d, respectively). The nucleoli are devoid of Sp1 regardless of the duration of insulin treatment or the presence of high levels of Sp1 in the nucleus. In contrast to total Sp1, the levels of O-GlcNAc-specific immunoreactivity also rapidly (30 min) accumulates in the nucleus (middle panel, f) but decreases at 60 min (middle panel, g) and even more at 240 min (middle panel, h). The negative nucleolar staining obtained with anti-Sp1 antibodies is not as obvious with anti-O-GlcNAc antibodies. Co-localization of Sp1 and O-GlcNAc residues demonstrates that O-GlcNAc-modified Sp1 initially (30 min) increases followed by a clear, steady decrease at 60 and 240 min (lower panel, j, k, and l, respectively). It also demonstrates that Sp1 is not homogeneously distributed throughout the nucleus, being absent from the nucleolus and from the nuclear periphery (seen as a rim of blue-purple fluorescence), and is most likely also absent in the nuclear membrane (lower panel, j and k). Magnification is × 200. B: insulin steadily enhances the phosphorylation of Sp1 for up to 240 min. H-411E cells were reacted with anti-Sp1 (green) or anti-phosphoserine (red) antibodies prior to their exposure to insulin (0 min) or 30, 60, and 240 min post-insulin treatment. Anti-Sp1 antibodies demonstrate the pattern shown in A. After insulin treatment, there is a steady increase in phosphorylated residues, first prominently seen in the nucleolus (middle panel, f), then in the nucleolus and nucleus (middle panel, g), and finally, in the nucleolus, nucleus, and cytoplasm (middle panel, h). There is a conspicuous absence of Sp1-specific staining in the nucleus (upper panel, b, c, and d). Unlike the levels of O-GlcNAc-modified Sp1 that initially (30 min) increased followed by a steady decrease at 60 and 240 min (Fig. 1A), there was continued accumulation of phosphorylated Sp1 in insulin-treated H-411E cells (lower panel, j, k, and l). Magnification of all other images is × 200.

3644 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 281 • NUMBER 6 • FEBRUARY 10, 2006

VOLUME 281 • NUMBER 6 • FEBRUARY 10, 2006
ammonium acetate (Fig. 2A). The amount of immunoreactive Sp1 in the cytoplasm, there is insignificant change in cytoplasmic accumulation of Sp1 following insulin treatment (Fig. 1A, b–d); in contrast, insulin treatment greatly enhanced the accumulation of nuclear Sp1. The nucleoli are consistently devoid of immunoreactive Sp1 regardless of the treatment. It is also evident that insulin induced a brisk O-GlcNAcylation of many cytoplasmic and nuclear proteins; O-GlcNAc-specific blue fluorescence staining peaked at 30 min after insulin treatment but declined thereafter at 60 and 240 min. Co-localization of Sp1 (green) and O-GlcNAc (blue) can be readily appreciated as turquoise immunofluorescence (blue + green). An increase in the O-glycosylated Sp1 in the nuclei of insulin-treated H-411E cells is seen at 30 min with a progressive decrease in turquoise immunofluorescence at 60 and 240 min. Thus, the pattern of accumulation of O-GlcNAc-modified Sp1 and the total Sp1 in response to insulin treatment is very different, since there is sustained enhancement of total Sp1 as judged by intense green fluorescence in the nuclei of insulin-treated cells. Interestingly, although abundant blue fluorescence representing O-GlcNAc-modified proteins can be detected at or near the nuclear membrane, this location is particularly devoid of O-glycosylated Sp1 (turquoise). Similarly, the nucleolus is also devoid of O-GlcNAc-modified proteins as well as Sp1, regardless of whether or not it is glycosylated (Figs. 1 and 3).

We also stained H-411E cells with anti-phosphoserine antibody, followed by red fluorescence-conjugated secondary antibody. As we have observed repeatedly, insulin stimulated accumulation of total Sp1 over the 240-min duration of treatment, as judged by increased accumulation of green fluorescence. Additionally, insulin treatment steadily enhanced the staining for anti-phosphoserine antibodies as detected with secondary antibody tagged with red fluorescence (Fig. 1B). Thus, there is a generally increased accumulation of phosphoserine-containing proteins in insulin-treated cells. However, insulin also specifically stimulated phosphorylation of Sp1 as judged by co-localization of Sp1-specific (green) and phosphoserine-specific (red) staining that is visible as yellow fluorescence. Interestingly, although phosphoserine-specific immunoreactivity abounds in the cytoplasm of insulin-treated H-411E cells (Fig. 1B, g and h), there is no detectable phosphorylated Sp1 in the cytoplasm. In contrast, the insulin-induced accumulation of phosphorylated Sp1 in the nucleus continued to increase over the 240-min time period. Finally, as opposed to what we observed for O-GlcNAc staining, the phosphoserine-specific antibody strongly stained the nucleoli (Fig. 1B, k and l). Thus, although nucleoli are characteristically devoid of Sp1, these organelles are significantly enriched in phosphoserine-containing immunoreactivity (Fig. 1B, k and l).

**Temporal Dynamics of Accumulation of O-Glycosylated and Phosphorylated Sp1 and the Steady State Levels of CaM mRNA in H-411E Cells**—To study the relationship between O-glycosylation and phosphorylation of Sp1, H-411E hepatoma cells were exposed to insulin for 0, 30, and 240 min, and the levels of O-GlcNAc-modified and phosphorylated Sp1 were assessed by Western blot analysis (Fig. 2A). The quantification of O-GlcNAc and phosphorylated Sp1 as probed with specific antibodies on Western blots shows that O-GlcNAc-Sp1 increased significantly at 30 min (p < 0.03), whereas phosphorylated-Sp1 was negligible (p = not significant) at 30 min but continued to increase through 240 min (p < 0.03). In contrast, O-GlcNAc-Sp1 was on the decline by 240 min (p < 0.04). The quantitative Western blot data presented in Fig. 2A are in agreement with the results of confocal microscopy that suggest a reciprocal relationship between O-glycosylation and phosphorylation of Sp1 after insulin exposure. Regulation of CaM gene expression is a key read-out of insulin action in H-411E cells (23–25). Therefore, we assessed the temporal relationship of the two posttranslational modifications of Sp1 to the changes in the steady state levels of CaM mRNA. We treated H-411E liver cells with insulin and harvested total RNA at 0 min, 30 min, 4 h, and 8 h. As shown in Fig. 2B, CaM mRNA levels increased steadily with time: 0 min, 0%; 30 min, 15%; 4 h, 64%; and 8 h, 100%, respectively.

**Effect of STZ on the Subcellular Distribution of O-Glycosylated and Phosphorylated Sp1 in the Presence of Insulin: Studies Using Confocal Microscopy**—In order to visualize the distribution of phosphorylated Sp1 in relation to glycosylated Sp1, we treated H-411E cells with STZ in the presence of insulin for 4 h to produce hyperglycosylation of Sp1 and then studied the subcellular localization of phosphorylated Sp1. Fig. 3, A and B, illustrate the localization of total Sp1, O-GlcNAc–Sp1, and phosphorylated Sp1 as assessed by staining with specific antibodies and confocal microscopy in H-411E hepatoma cells treated with insulin and STZ. STZ inhibits O-GlcNAcase, the enzyme responsible for removing O-GlcNAc from O-glycosylated proteins. Consistent with earlier observations (7, 8), Fig. 3A shows that insulin stimulates synthesis of total Sp1 and the accumulation of O-GlcNAc-modified proteins; insulin also stimulates O-GlcNAc–Sp1 as judged by the co-localization of O-GlcNAc- and Sp1-specific staining. The O-GlcNAc staining is enhanced in STZ-treated cells, and the levels of O-GlcNAc–Sp1 are
Insulin Regulates Sp1 by O-Glycosylation/Phosphorylation

FIGURE 3. A, accumulation of enhanced levels of O-GlcNAc-modified Sp1 in STZ-treated H-411E cells after insulin treatment. H-411E cells were treated with insulin alone or STZ alone or co-treated with insulin plus STZ for 4 h and reacted with anti-Sp1 (green) and anti-O-GlcNAc (blue) antibodies. Cells treated with insulin alone had more nuclear Sp1 (upper panel, c) than untreated controls (upper panel, a) but less than insulin-treated cells (upper panel, b). Cells treated with insulin plus STZ had more demonstrable cytoplasmic Sp1 than any other treatment, suggesting a combination of increased synthesis and decreased transport. O-GlcNAc residues were not present in the nucleolus with any treatment. However, cells treated with insulin plus STZ had demonstrable cytoplasmic Sp1 and O-GlcNAc residues (middle panel, h). Co-localization of Sp1 and O-GlcNAc-specific immunoreactivity shows that the nuclear periphery (blue rim) is devoid of Sp1 (lower panel, j, k, and l), regardless of treatment. O-GlcNAc-modified Sp1 continues to persist in the nucleus and cytoplasm of STZ-treated and insulin plus STZ-co-treated cells (lower panel, k and l). This is in contrast to the decreased amount of O-glycosylated Sp1 in cells treated with insulin alone (lower panel, f). Magnification is ×200. B, enhanced accumulation of phosphorylated Sp1 in STZ-treated H-411E cells is inhibited by insulin treatment. H-411E cells treated with insulin alone, STZ alone, or insulin plus STZ for 4 h were reacted with anti-Sp1 (green) and anti-phosphoserine (red) antibodies. The distribution of Sp1 immunoreactivity is similar to the distribution displayed in the previous figures (A, upper panel, a–d). Abundant phosphoserine-specific staining can be observed in the nucleus and cytoplasm of insulin-treated cells (middle panel, f). Treatment with STZ (middle panel, g) resulted in more phosphoserine-specific staining in nuclei and cytoplasm than in untreated cells (middle panel, e) but less than that seen in insulin-treated cells (middle panel, f). Combined treatment with insulin and STZ (middle panel, h) resulted in reduced levels of phosphoserine-specific staining in nuclei and cytoplasm than with either agent alone (middle panel, f and g). Magnification of a, e, and f is ×40. Magnification of all other images is ×200.

Effect of STZ on O-Glycosylation and Phosphorylation of Sp1 and the Steady State Levels of CaM mRNA in Insulin-treated H-411E Cells—To further study the reciprocal relationship between O-glycosylation and phosphorylation of Sp1 in response to insulin, we quantified levels of phosphorylated Sp1 in cells treated with STZ to inhibit O-GlcNAcase and enhance glycosylation of Sp1. Fig. 4A illustrates the steady state levels of immunoprecipitated Sp1 from H-411E hepatoma cells exposed to insulin in the presence or absence of STZ. Total immunoprecipitable Sp1 was subjected to Western blot using anti-O-GlcNAc- and anti-phosphoserine antibodies. We found that insulin consistently stimulated both O-glycosylation and phosphorylation of Sp1. However, when cells were treated with insulin and STZ together, O-glycosylated Sp1 was markedly increased (p < 0.05), with a concomitant decline in the steady state levels of phosphorylated Sp1 (p < 0.03). These data are consistent with the concept that STZ prevented removal of O-GlcNAc from the serine(s) on Sp1, and the O-GlcNAc-modified serines could not then be phosphorylated. We further assessed the relationship between O-glycosylation and phosphorylation of Sp1 and the CaM gene transcription. We treated H-411E cells with STZ to induce hyperglycosylation of Sp1 and then examined the steady state levels of CaM mRNA. Fig. 4B illustrates that, consistent with earlier published data, insulin alone stimulated CaM mRNA, but co-treatment of cells with STZ and insulin significantly decreased the levels of CaM mRNA as compared with CaM mRNA levels seen in cells treated with insulin alone (p < 0.007).

Temporal Analysis of Insulin-mediated Glycosylation and Phosphorylation of Sp1 by MALDI-TOF MS—We extended our observations of a reciprocal relationship between O-glycosylated and phosphorylated...
Sp1 in response to insulin as judged by immunohistochemistry and confocal microscopy by directly analyzing Sp1 by MALDI-TOF MS. H-411E liver cells were treated with or without insulin (10,000 microunits/ml) for 4 h in the presence of STZ (5 mM) as described under “Experimental Procedures.” The immunoprecipitated Sp1 was subjected to SDS-PAGE and Western blot analysis using either anti-O-GlcNAc antibody or anti-phosphoserine antibody. Densitometry values of the Western blots from multiple experiments are presented. Arbitrary units shown on the y axis are net change (stimulated minus basal). Values are shown as mean ± S.E. Number of replicates, n = 3. *, p < 0.05 versus insulin; **, p < 0.03 versus STZ plus insulin. B, effect of STZ on CaM mRNA in the presence of insulin. Quantification of Northern blots by densitometry for CaM mRNA is reported with average values ± S.E. The number of replicates is 3. Data shown are net change (stimulated minus basal). *, p < 0.007 versus insulin.

FIGURE 4. Effect of insulin plus STZ co-treatment on O-glycosylation and phosphorylation of Sp1 and on the steady state levels of CaM mRNA. A, O-glycosylation and phosphorylation of Sp1 after exposure to insulin, STZ, or insulin plus STZ. H-411E cells were treated with or without insulin (10,000 microunits/ml) for 4 h in the presence of STZ (5 mM) as described under “Experimental Procedures.” The immunoprecipitated Sp1 was subjected to SDS-PAGE and Western blot analysis using either anti-O-GlcNAc antibody or anti-phosphoserine antibody. Densitometry values of the Western blots from multiple experiments are presented. Arbitrary units shown on the y axis are net change (stimulated minus basal). Values are shown as mean ± S.E. Number of replicates, n = 3. *, p < 0.05 versus insulin; **, p < 0.03 versus STZ plus insulin. B, effect of STZ on CaM mRNA in the presence of insulin. Quantification of Northern blots by densitometry for CaM mRNA is reported with average values ± S.E. The number of replicates is 3. Data shown are net change (stimulated minus basal). *, p < 0.007 versus insulin.
**Insulin Regulates Sp1 by O-Glycosylation/Phosphorylation**

**TABLE 1**

| Determined mass | Theoretical mass | Position | Peptides |
|-----------------|------------------|----------|----------|
| 563.27          | 563.24           | 612–616  | DSEGR    |
| 617.24          | 617.28           | 617–623  | GSGDPGK  |
| 613.34          | 613.34           | 641–645  | TSHLR    |
| 672.36          | 672.33           | 680–685  | THTGEC   |
| 685.33          | 686.34           | 699–704  | SDSLHK   |

**DISCUSSION**

We have previously demonstrated that Sp1, an obligatory transcription factor, stimulates CaM gene transcription in response to insulin (5, 6). Insulin stimulates synthesis, O-glycosylation, and phosphorylation of Sp1, and this sequential O-glycosylation and phosphorylation of Sp1 enables it to activate CaM gene transcription (6–8). The question of the upstream signals involved in the sequential changes in the enhanced rate of biosynthesis and posttranslational modifications of Sp1 in response to insulin has received only limited attention to date. Based on our published studies using specific inhibitors of signaling (19, 26), we believe that post-insulin receptor activation mechanism that leads to phosphorylation of Sp1 precedes phosphorylation of Sp1 localized in the nucleus and not in the nucleolus or nuclear periphery. The confocal microscopic observations demonstrate that a temporal reciprocal relationship exists between O-glycosylated-Sp1 and phosphorylated Sp1 in the H-411E cells treated with insulin and that these findings are corroborated by the MALDI-TOF MS data. Thus, O-glycosylation of Sp1 precedes phosphorylation, and the MALDI-TOF MS indicates that the O-GlcNAc and phosphate moieties are both present at different times on identical serine site(s) of Sp1. Furthermore, we find a close correlation of CaM gene transcription with the steady increase in phosphorylation of Sp1 following insulin stimulation. Studies with STZ extend these observations and show that the removal of O-GlcNAc residues from Sp1 is required prior to phosphorylation and that this dynamic interaction occurs in the nucleus. An alternative but less likely interpretation would be that O-glycosylation and phosphorylation of Sp1 take place in the cytoplasm but that the transit to the nucleus is so rapid as to make the cytoplasmic residues virtually undetectable.

O-GlcNAc is a modification of serine and threonine residues of nuclear and cytoplasmic proteins with O-linked β-N-acetylglucosamine. Most of the O-GlcNAcylated proteins can be phosphorylated (9). Since O-GlcNAc and O-phosphate may share the same or adjacent serine or threonine sites on a protein, these modifications may be reciprocally regulated to modulate intracellular signaling and gene expression (10, 12).

O-GlcNAc and O-phosphate modifications have been identified on many transcription factors (11, 27). Sp1 is extensively modified by O-GlcNAc (3, 9) and phosphate (15, 28, 29) on multiple serine and threonine residues. Work from Kudlow’s laboratory (30) has identified SerSerSerSerSer484 in the activation domain of Sp1 as one of the most critical sites for Sp1 activity and a likely site for O-glycosylation. Some investigators have shown that a reciprocal relationship exists between O-glycosylation and phosphorylation of Sp1 (7, 31), whereas others reported that Sp1 O-glycosylation remained unchanged with increased phosphorylation of Sp1 (28). We show that in insulin treated H-411E liver cells, O-GlcNAc-Sp1 rapidly (30 min) accumulated in the nucleus and to a lesser extent in the cytoplasm but slowly declined, whereas phosphorylation of Sp1 progressively increased in the nucleus with time, reflecting a temporal reciprocal existence between O-glycosylation and phosphorylation of Sp1. Interestingly, no phosphorylated Sp1 was detected in the cytoplasm, although phosphoserine-specific immunoreactivity increased in the cytoplasm over time with insulin. Since the total nuclear Sp1 and phosphorylated Sp1 increased progressively over time with insulin stimulation, it is likely that dephosphorylated Sp1 may be phosphorylated in the nucleus. Kamemura and Hart (15) recently proposed that Thr458 glycosylation of c-Myc in HL-60 cells occurs prior to its phosphorylation and that the interplay between these modifications is thought to modulate subcellular trafficking of c-Myc and other proteins (10, 12). It is worth noting that although nucleoli have abundant immunoreactivity of serine-phosphorylated proteins, they are devoid of Sp1 (Figs. 1, A and B, and 3, A and B).

Confocal microscopy revealed that insulin alone increased phosphorylated Sp1 in the nucleus and not in the cytoplasm, but the combination of STZ plus insulin decreased phosphorylated Sp1 significantly in the nucleus. STZ plus insulin together, strongly stimulated O-GlcNAcylation of Sp1 (insulin by stimulating OGt and STZ by inhibiting O-GlcNAcase) both in the nucleus and the cytoplasm. In the presence of STZ + insulin, the serine sites to be phosphorylated would remain O-GlcNAc-modified and, therefore, not available for phosphorylation, and predictably, reduced phosphorylated Sp1 was seen under these con-
Insulin Regulates Sp1 by O-Glycosylation/Phosphorylation

In a number of investigations, Hart’s laboratory (10, 15, 36) has proposed an orchestrated reciprocal shuttle between O-GlcNAc and phosphorylated Sp1 as a major regulatory mechanism, translating signal transduction effects from the plasma membrane to the gene. We have shown previously that insulin stimulates CaM gene expression (23). Following insulin stimulation, it was observed that CaM gene transcription increased in a time-dependent manner. The level of CaM mRNA was low at 30 min, gradually increasing to significant levels at 240 min. Interestingly, we also demonstrated that O-glycosylated Sp1 peaked early but later declined after insulin stimulation, whereas phosphorylation of Sp1 steadily rose, becoming significant at 4 h. This progressive increase in phosphorylation of Sp1 most closely correlated with the steady increase in CaM mRNA after insulin (Fig. 2, A and B). Furthermore, in the presence of STZ, there was a significant reduction in the insulin-mediated increase of CaM gene transcription accompanied with a decrease in phosphorylated Sp1 (Fig. 4, A and B). Thus, phosphorylation of Sp1 appears to correlate more directly with the kinetics of enhanced CaM gene transcription. Studies have shown that changes in the phosphorylation state of Sp1 regulate activity of this transcription factor (28, 37). Other investigators have suggested that a balance between phosphorylation and O-glycosylation of proteins is required for regulatory mechanisms (33). Our data indicate that when the ratio of O-GlcNAc-Sp1 to phosphorylated Sp1 is greater than 1, intracellular recompartmentalization of Sp1 and initiation of CaM gene transcription occurs, but when the same ratio is less than 1, CaM gene transcription is markedly enhanced.

Whereas our data convincingly show that O-GlcNAc residues have to be removed from Sp1 before it could be phosphorylated, we do not know whether insulin directly modulates the activities of both OGT and O-GlcNAcase in a temporal sequence as predicted from our observations. We speculate, however, that insulin orchestrates a fine balance between OGT and O-GlcNAcase activities in a time-dependent manner. We have shown earlier that insulin regulates the activity of OGT (7). However, the precise molecular mechanisms underlying the apparent sequence of regulation of specific OGT, O-GlcNAcase, and protein kinase(s) that modify Sp1 and reprogram gene expression in response to insulin remain to be elucidated.

We may ask whether insulin-mediated reprogramming of gene expression via Sp1 is unique to insulin or if such a mechanism is also shared by insulin-like growth factor (IGF-1). This is a relevant question. Since IGF-1 binds to the IGF receptor and insulin receptor, and insulin binds to both the insulin receptor and IGF receptor, perhaps some features of insulin and IGF-1 signaling are shared. Evidence emerging from a number of recent studies indicates that IGF-1 may also elicit Sp1-mediated altered regulation of gene expression. For instance, Li and colleagues (38) analyzed regulation of global gene expression by IGF-1 by DNA microarrays in cardiac muscle and demonstrated that Sp1-binding motifs were a hallmark of the promoters of many IGF-1-inducible genes. These authors also directly tested a subset of IGF-1-inducible genes (such as cyclin D3 and GLUT-1) and demonstrated that the IGF-1 response of these genes was critically dependent on Sp1. IGF-1-induced activation of peptide YY promoter was also shown to be dependent on the activity of Sp1 (39). Even more importantly, the promoter of IGF-1 contains Sp1 binding motifs that are crucial for its regulation by insulin (40). Whether autocrine regulation of IGF-1 also involves the action of Sp1 remains to be demonstrated. We should stress here that although the rate of biosynthesis and activities of Sp1 were enhanced by IGF-1 treatment, the authors of the above mentioned studies did not report if Sp1 underwent specific changes in the posttranslational modifications under these conditions. Finally, we should point out that we have not directly tested whether IGF-1 also stimulates CaM gene expression in the H411E hepatoma cells. However, we speculate that IGF-1 treatment is likely to mimic insulin and stimulate CaM gene expression.

In summary, our confocal microscopy data support and extend our biochemical data on the subcellular localization of Sp1 demonstrated earlier (8). We show that insulin stimulates O-glycosylation of Sp1 that promotes its migration to the nucleus, where O-GlcNAc sites are removed and replaced by phosphate. Thus, the sequential and coordinated modification of Sp1 O-GlcNAcylation and phosphorylation appear to play a central role in determining the ability of insulin to enhance transcription of the CaM gene.

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Insulin Regulates Sp1 by O-Glycosylation/Phosphorylation