O-glycosylation of Sp1 and transcriptional regulation of the calmodulin gene by insulin and glucagon

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Majumdar, Gipsy, Ashley Harmon, Rosalind Candelaria, Antonio Martinez-Hernandez, Rajendra Raghow, And Solomon S. Solomon. O-glycosylation of Sp1 and transcriptional regulation of the calmodulin gene by insulin and glucagon. Am J Physiol Endocrinol Metab 285: E584–E591, 2003; 10.1152/ajpendo.00140.2003.—Both insulin and glucagon stimulate steady-state levels of Sp1 transcription factor, but only insulin stimulates transcription of the calmodulin (CaM) gene in liver. Because O-glycosylation of Sp1 by O-linked N-acetylglucosamine (O-GlcNAc) is thought to regulate its ability to activate transcription, we assayed the levels of Sp1 with anti-Sp1 and anti-O-GlcNac antibodies in Western blots by use of extracts of H-411E liver cells treated with insulin (10,000 μU/ml) or glucagon (1.5 × 10−5 M). We also assessed subcellular localization of the native and glycosylated Sp1 in H411E cells treated with either hormone in the presence of deoxynorleucine (DON, an indirect inhibitor of O-glycosylation) or streptozotocin (STZ, an indirect stimulator of O-glycosylation). Insulin stimulated both total and O-GlcNac-modified Sp1 primarily in the nucleus and induced CaM gene transcription (P < 0.0001). In contrast, glucagon promoted accumulation of Sp1 in the cytoplasm but not the nucleus, without significantly stimulating (P = not significant) either its O-glycosylation or transcription of the CaM gene. DON inhibited O-glycosylation of Sp1 and its ability to migrate to the nucleus and transactivate CaM gene transcription. In contrast, cotreatment of cells with STZ and glucagon enhanced O-glycosylation of Sp1, promoting its migration to the nucleus and resulting in increased CaM gene transcription. Thus O-glycosylation of Sp1 by insulin, but not glucagon, apparently enhances its (Sp1) nuclear recruitment and results in activation of CaM gene transcription.

Sp1 transcription factor; calmodulin gene transcription; liver cells; diabetes mellitus

STUDIES OF INSULIN ACTION on signal transduction have focused primarily on membrane events (22). However, the importance of insulin action in the nucleus and its ability to regulate gene transcription is becoming increasingly recognized (20). The mechanistic basis of altered gene expression in response to insulin remains poorly defined. We have recently described that, although both insulin (13) and glucagon (11) stimulate Sp1 synthesis in H-411E liver cells in culture, only insulin promoted calmodulin (CaM) gene transcription. Furthermore, although insulin and glucagon individually stimulated Sp1 (insulin more than glucagon), together they did not; in fact, glucagon inhibited the stimulatory effect of insulin, thus mimicking the well-known counterregulatory actions of these hormones in vivo (11).

O-glycosylation [covalent addition of O-linked N-acetylglucosamine (O-GlcNAc)] is an abundant posttranslational modification found on a number of nuclear and cytoplasmic proteins (6, 21). O-glycosylation is thought to be a dynamically regulated process much like phosphorylation, and there is mounting evidence that posttranslational addition of O-GlcNAc to transcription factors may be directly involved in regulating their function (6, 21). Recently, considerable interest has been focused on the role of O-glycosylation in modulating Sp1-dependent transcription. However, the precise action of O-glycosylation on Sp1 is far from clear. Some investigators have shown that O-glycosylation activates Sp1-dependent transcription without altering its DNA binding (5, 9). On the other hand, it has also been reported that O-glycosylation of Sp1 inhibited its ability to activate transcription (2, 5). We have recently shown (11) that insulin, but not glucagon, O-glycosylates Sp1 and significantly enhances its ability to activate transcription of the CaM gene.

Herein, we report that insulin and glucagon differentially affect O-glycosylation of Sp1. We also demonstrate that insulin stimulates glycosylation of Sp1 and apparently facilitates its movement from cytoplasm to nucleus, where it activates transcription of the CaM gene. In contrast to insulin, treatment of H411E cells with glucagon apparently increases accumulation of unglycosylated Sp1 in the cytoplasm and, hence, does not promote transcriptional activation of the CaM gene.

MATERIALS AND METHODS

Chemicals. Insulin, glucagon, and protein standards were obtained from Sigma (St. Louis, MO). Nuclear isolation kit,
deoxyribo nucleic acid (DON), 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-linked GlcNAc antibody was obtained from Affinity BioReagents (Golden, CO). Protein A Sepharose, Bio-Rad protein assay reagent, and kaleidoscope protein molecular weight markers were obtained from Bio-Rad laboratories (Hercules, CA).

Cell culture. Minimally deviant H-411E rat hepatoma cells were obtained from American Type Culture Collection and were grown in Eagle's minimum essential medium (EMEM) supplemented with 1% glutamine, 1% nonessential amino acids, 1% streptomycin-penicillin, and serum (10% calf serum, 5% fetal bovine serum). Cell cultures were incubated at 37°C in 5% CO2-95% air with tissue culture medium and then scraped and lysed to prepare nuclear, membrane, and cytosolic fractions by a modification of the procedure described by Smaoke and Solomon (17). The nuclear pellet was obtained by low-speed centrifugation (500 g at 4°C) for 15 min. The pellet was washed once in the EZ lyse buffer and then resuspended in a hypertonic buffer (10 mHEPES, pH 7.4, 0.42 M NaCl, 1.5 mM MgCl2, 2.5% glycerol, 1 mM EDTA, 1.5 mM DTT, and the mixture of protease inhibitors). The nuclei were allowed to swell for 30 min at 4°C, and a clear nuclear extract was obtained by centrifugation at 100,000 g for 20 min at 4°C in a Beckman ultracentrifuge. The supernatant from the initial low-speed centrifugation (500 g) was further spun at 100,000 g for 20 min to obtain both a pellet of the crude membrane fraction and the soluble cytosolic fraction. Cross contamination of various cellular fractions was not assessed in these particular experiments but has been done by us in previous experiments and is ~10%-20% (17). To identify the effects of insulin or glucagon on glycosylated Sp1, nuclear, membrane, and cytosolic fractions (500 μg total cellular protein/sample) were subject to immunoprecipitation with polyclonal anti-Sp1 antibody and electrophoresed on 7.5% SDS polyacrylamide gel. After Western blotting, the membranes were probed with anti-O-linked GlcNAc monoclonal antibody and then stripped and reprobed with anti-Sp1 polyclonal antibody.

Immunocytochemical staining. Cells were grown in RS coated glass chamber slides (Nalge Nunc International, Naperville, IL). After achieving 60% confluence, cells were treated overnight (12–16 h) with serum-free medium containing combinations of insulin, glucagon, STZ, and DON, as described in previous experiments. In the present experiments using STZ and DON alone or in combination with insulin or glucagon, cells were treated with glucose-free and serum-free media for 12–16 h. In all these experiments, the cells had already been treated for 12–14 h in serum-free medium and then again with hormone alone or hormone plus selected inhibitor overnight (12–16 h). Concentrations of agents used were as follows: insulin, 10,000 μU/ml; glucagon, 1.5 × 10^{-5} M; DON, 100 μM; and STZ, 5 mM. Cells were washed with PBS and immediately fixed with 10% neutralized formalin. Immunocytochemical staining was performed with anti-Sp1 antibody (11). The signal was developed by horseradish peroxidase-conjugated second antibody employing a 3′,3′-diaminobenzidine tetrahydrochloride-plus kit from Zymed Laboratory (San Francisco, CA).

Studies performed using DON and STZ to assess insulin or glucagon stimulation of O-glycosylated Sp1. Confluent H-411E liver cells were cultured in serum-free media for 12–16 h and then treated with or without STZ (5 mM) in glucose-free and serum-free media overnight (14–16 h) as described above. After overnight incubation, the cells were exposed to insulin (10,000 μU/ml) for 4 h. In addition, STZ-free cells (without insulin) were exposed to DON (100 μM) for 1 h and
insulin plus DON for an additional 4 h. DON inhibits glycosylation indirectly by inhibiting glutamine-fructose-6-phosphate aminotransferase, a key enzyme in the hexosamine pathway that produces UDP-GlcNAc. STZ, an analog of GlcNAc, stimulates glycosylation by inhibiting the enzyme glucosaminidase, which removes GlcNAc moieties from protein.

In another set of experiments, confluent H-411E cells were cultured in serum-free medium for 12–16 h and then treated with or without STZ (5 mM) in glucose-free and serum-free media overnight (14–16 h), as described previously. After overnight incubation, the cells were exposed to glucagon (1.5 x 10^-6 M) for 4 h. In addition, STZ-free cells (without glucagon) were exposed to DON (100 μM) for 1 h and glucagon plus DON for an additional 4 h.

After these treatments, 500 μg of total proteins extracted from the cells were subject to immunoprecipitation to assess both O-glycosylated and total Sp1 by Western blot. Blots were sequentially probed with anti-O-linked GlcNAc monclonal antibody, stripped, and reprobed with anti-Sp1 polyclonal antibody as previously described (11).

mRNA analysis. Cells were treated with insulin and glucagon alone and together, similar to the previous experiments. Total RNA was collected and mRNA analysis performed using a cDNA probe for CaM, as described previously (11).

Statistical analysis. Bands in the X-ray films were scanned and quantified using the Quantity One software program from Bio-Rad Laboratory 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, this is so stated; if unpaired, it is referred to simply as Student’s t-test.

RESULTS

Effects of insulin and glucagon on subcellular distribution of Sp1 and O-GlcNAc Sp1 in H-411E hepatoma cells. Because intracellular movement of many proteins is significantly influenced by posttranslational O-glycosylation and/or phosphorylation, we assessed whether or not the subcellular localization of Sp1 was affected by either insulin or glucagon treatment. Tables 1 and 2 present the subcellular distribution of total and O-GlcNAc-modified Sp1 after treatment with insulin or glucagon. Consistent with our previous observations (11), both insulin (+145%) and glucagon (+125%) increased the total amount of immunoreactive Sp1 in H411E cells. However, we observed that the subcellular distribution of Sp1 in response to each hormone is clearly different (Table 1). Although there was a slight increase in cytosolic and membrane Sp1 in response to insulin, insulin predominantly stimulated the Sp1 content of the nucleus (+88%). This is in contrast to glucagon (P = not significant [NS]), which did not significantly increase Sp1 content in either nucleus or plasma membrane. Most of the glucagon-stimulated Sp1 (+84%) was found in the cytoplasm.

Table 2 illustrates the distribution of glycosylated Sp1 among the three subcellular fractions after insulin and glucagon treatment. The data show that insulin-treated cells preferentially accumulated most of the glycosylated Sp1 in their nuclei (P < 0.02). In contrast, glucagon did not significantly promote glycosylation of Sp1 in nuclear, membrane, or cytoplasmic fractions. It appears, therefore, that insulin markedly increased Sp1 (P < 0.02) and O-GlcNAc Sp1 (P < 0.01) in the nucleus, whereas glucagon significantly increased neither O-glycosylation of Sp1 (P = NS) nor its nuclear translocation.

Immunocytochemical studies of effects of insulin or glucagon on intracellular distribution of Sp1 and O-glycosylated proteins. To directly visualize the intracellular distribution of Sp1, H411E cells grown in glass chamber slides were exposed to either insulin or glucagon and then stained with anti-Sp1 antibody or anti-O-GlcNAc antibody followed by horseradish peroxidase-conjugated second antibody. As shown in Fig. 1, insulin and glucagon both increased Sp1 content of H411E cells. However, anti-O-GlcNAc antibody staining revealed that cells treated with insulin had increased glycosylation of proteins in the nucleus (Fig. 2d). The weak overall glycosylation in glucagon...
treated H411E cells, as judged by staining with anti-O-GlcNAc antibodies (Fig. 2b), was similar to that in untreated controls. Furthermore, when cells were cotreated with insulin and an inhibitor of O-glycosylation, DON, the nuclear staining was clearly decreased (Fig. 1f). In contrast, when STZ, a stimulator of O-glycosylation, was added with insulin to liver cells, both Sp1 (Fig. 1i) and anti-O-GlcNAc (Fig. 2f) staining shifted from cytoplasmic components back to the nucleus (Fig. 2f). Because glucagon stimulates primarily unglycosylated Sp1, we added STZ to cells treated with glucagon to see whether promotion of glycosylation by STZ could reverse the effects of glucagon alone. Consistent with our expectations, when STZ was added to glucagon-treated cells, Sp1 staining shifted from cytoplasm to nucleus and there was a dramatic increase in its glycosylation (Fig. 1, g and h). Thus, by inhibiting O-glycosylation of Sp1, DON counteracts the effect of insulin, so it behaves like glucagon, whereas glucagon in the presence of STZ, which stimulates O-glycosylation of Sp1 and its nuclear translocation, mimics the effect of insulin.

Effects of DON and STZ on O-glycosylation of Sp1 stimulated with either insulin or glucagon. Studies detailed in Figs. 3 and 4 demonstrate the ability of DON to inhibit, and of STZ to enhance, O-glycosylation in H411E cells treated with either insulin or glucagon. A representative Western blot of O-GlcNAc-modified Sp1 and its quantification from multiple Western blot experiments are shown in Fig. 3, A and B, respectively. O-glycosylation and nuclear localization of Sp1 in insulin-treated cells was significantly stimulated (P < 0.005), as shown in Tables 1 and 2. Although addition of DON to insulin-treated cells inhibited glycosylation of Sp1 (insulin + DON vs. insulin alone, P < 0.01), cotreatment of cells with insulin and STZ stimulated glycosylation of Sp1 (insulin + STZ vs. insulin alone, P < 0.05).

Experiments shown in Fig. 4 demonstrate the effects of DON and STZ on glucagon stimulation of O-glyco-
sylation of Sp1 in H-411E cells. Figure 4A shows a typical Western blot for Sp1 from H411E cells either treated with glucagon alone or simultaneously treated with glucagon and DON or glucagon and STZ. Figure 4B summarizes multiple experiments to quantify Sp1 from H411E cells exposed to glucagon in the presence of DON or STZ. These blots were reprobed with an anti-actin antibody, which showed that actin remained unchanged in the presence of either insulin or glucagon during the various treatments (data not shown). O-GlcNAc modification of Sp1 was only minimally stimulated by glucagon compared with control (P = NS). However, STZ significantly stimulated O-glycosylation of Sp1 in glucagon-treated H-411E cells (glucagon vs. glucagon + STZ, P < 0.05), whereas DON inhibited the O-glycosylation reaction. These data are consistent with the known actions of the two agents; thus DON inhibited and STZ stimulated O-GlcNAcation of Sp1. Furthermore, although glucagon stimulated total Sp1, it failed to significantly stimulate the steady-state levels of O-GlcNAc Sp1. The overall O-glycosylation of Sp1 in response to insulin and glucagon in the presence of DON and STZ was predictable. Apparently, the effects of these pharmacological agents overrode both insulin and glucagon alone with respect to their ability to modulate O-glycosylation of Sp1. We should note, however, that, regardless of treatment, in each case the status of O-glycosylation of Sp1 was totally consistent with the subcellular compartmentalization data; i.e., greater glycosylation led to higher nuclear translocation of Sp1 (Tables 1 and 2).

Effects of DON or STZ on insulin or glucagon stimulation of CaM mRNA in H-411E liver cells. Because we have previously shown that insulin, but not glucagon, treatment resulted in enhanced rates of CaM gene transcription, we were curious to assess whether or not O-glycosylation in response to insulin or glucagon, with or without inhibitors, would affect CaM gene expression. The rates of transcription of the CaM gene, as deduced by analysis of Northern blot data, in H411E cells treated with these hormones either alone or together with modifiers of O-glycosylation are shown in Tables 3 and 4. Consistent with previously published results, insulin alone stimulated CaM gene transcription (+85%, P < 0.0001), and addition of STZ further enhanced the insulin-mediated increase in CaM gene transcription (+184%, P < 0.06). The apparent minimal stimulation of CaM mRNA in response to glucagon was not statistically significant (+21%, P = NS). Treatment of H411E cells with DON by itself inhibited CaM gene transcription (−17%, P = NS). DON also blocked the enhanced steady-state levels of CaM mRNA in insulin-treated cells (−30%, P < 0.005). Similarly, although glucagon alone did not significantly stimulate,
cotreatment with STZ significantly stimulated steady-state levels of CaM mRNA (±162%, P < 0.04).

On the basis of these data, we may surmise that there is a direct correlation between glycosylation of Sp1 and the rate of CaM gene transcription. However, as noted earlier, these inhibitors are more general than specific and act “indirectly.”

DISCUSSION

Previous work from our laboratory (17, 18) has shown that insulin positively regulates transcription of the rat CaM gene. The basal insulin-responsive CaM promoter contains three Sp1-binding sites. To elucidate the mechanism of transcriptional regulation, rat hepatoma (H-411E) cells were transfected with DNA constructs containing CaM promoters coupled to a luciferase reporter and stimulated by insulin. Rat CaM I promoters containing all three Sp1 sites or truncated promoters with combinations of one to three Sp1 sites were studied in Sp1-deficient Drosophila SL2 cells with or without cotransfection with an Sp1 expression vector and challenged with insulin. Our promoter dissection experiments have demonstrated that Sp1 is obligatory for both basal and insulin activation of the CaM promoter. We found that a minimum of two of the three Sp1 sites in the CaM promoter, particularly the

### Table 3. Effects of DON or STZ on insulin stimulation of CaM mRNA in H-411E liver cells

<table>
<thead>
<tr>
<th>Control</th>
<th>Insulin</th>
<th>DON</th>
<th>Insulin + DON</th>
<th>STZ</th>
<th>Insulin + STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>185 ± 5%</td>
<td>108 ± 4%</td>
<td>156 ± 4%</td>
<td>179 ± 9%</td>
<td>284 ± 26%</td>
</tr>
<tr>
<td>P vs. Con</td>
<td>P vs. Con</td>
<td>P vs. Ins</td>
<td>P vs. Con</td>
<td>P vs. Ins</td>
<td></td>
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<tr>
<td>&lt; 0.0001</td>
<td>&lt; 0.005</td>
<td>&lt; 0.01</td>
<td>&lt; 0.06, NS</td>
<td>(n = 4)</td>
<td>(n = 5)</td>
</tr>
</tbody>
</table>

Denisomeric quantitation of autoradiographs of Northern blots for calmodulin (CaM) mRNA is reported with average values ± SE. DON, deoxynorleucine; STZ, streptozotocin. Data are normalized to control (Con, 100%).

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The ubiquitous transcription factor Sp1 is modified extensively by O-GlcNAc (9), but whether this modification regulates Sp1 function is controversial. Jackson and Tjian (9) were the first to propose that O-linked GlcNAc residues play a role in the mechanism of regulation of transcriptional activation of RNA polymerase II and that the same modification of Sp1 was functionally important, since blocking O-GlcNAc residues by wheat germ agglutinin inhibited Sp1-dependent transcription in vitro. Brownlee’s laboratory [Du et al. (2)] suggested that O-glycosylation of Sp1 markedly improved its ability to activate transcription. Others have suggested a role for O-GlcNAcylation in the assembly of the preinitiation complex in the transcriptional process (7, 26).

To explain the complex relationship of O-glycosylation and regulation of transcription, Kudlow’s laboratory [Han and Kudlow (5)] conducted investigations that initially supported the concept that O-GlcNAc-modified Sp1 was active and that the hyperglycosylation of Sp1 stabilized the protein; under conditions of glucose starvation, Sp1 was deglycosylated and thereby subjected to proteosomal degradation, leading to reduced gene transcription. However, these investigators later suggested that transcriptional repression mechanisms may also use O-glycosylation of Sp1 to turn off transcription (23, 24). Alternatively, there is strong evidence to indicate that Sp1 degradation is not dependent on the O-GlcNAc state of Sp1 but rather on the state of the proteosome. Sp1 degradation is blocked when O-GlcNAc levels increase in the cell because its proteosomal degradation is blocked. With more Sp1 accumulating, it (Sp1) binds to its target gene(s) and then is modified or unmodified as the local gene environment dictates (Kudlow J, personal communication).

Hence, O-GlcNAc may positively regulate some genes, while negatively regulating others (21, 24). Thus contradictory conclusions from different laboratories might be explained by a model in which O-glycosylated-Sp1 binds to the DNA, is then deglycosylated and phosphorylated, and goes on to activate gene transcription (25).

Several researchers have proposed that O-GlcNAc modification may also play a role in nuclear transport. The nucleus represents one of the sites of greatest concentration of O-GlcNAc-proteins (8), and many O-GlcNAcylated proteins shuttle from the cytoplasm to the nucleus (1, 3). Our data indicate that insulin stimulates the accumulation of both total and glycosylated Sp1 in the nucleus with a concomitant decrease in the total and O-GlcNAc-modified Sp1 in the cytoplasm. Subcellular distribution of glycosylated Sp1, as deduced biochemically as well as by immunohistochemical staining, strongly suggests that glycosylation is involved in the translocation of Sp1 to the nucleus. Kearse and Hart (10) have shown that activation of lymphocytes results in decreased levels of O-GlcNAc proteins in the cytoplasm with a concomitant increase of glycosylated proteins in the nucleus. Glucagon does not O-glycosylate Sp1, apparently reducing its mobility and leaving it to accumulate in the cytoplasm. STZ can overcome this effect of glucagon by enhancing glycosylation and nuclear translocation of Sp1, resulting in a significant increase in CaM gene transcription.

The synergistic effect of STZ with glucagon is not clear. STZ, an analog of GlcNAc, is known to inhibit β-N-acetylglucosaminidase (O-GlcNacase), an enzyme that cleaves GlcNAc residues from intracellular pro-

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Table 4. Effects of DON or STZ on glucagon stimulation of CaM mRNA in H-411E liver cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Glucagon</th>
<th>DON</th>
<th>Glucagon + DON</th>
<th>STZ</th>
<th>Glucagon + STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon</td>
<td>100%</td>
<td>121±7%</td>
<td>83±8%</td>
<td>104±4%</td>
<td>134±13%</td>
<td>262±27%</td>
</tr>
<tr>
<td>P vs. Con</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.04</td>
</tr>
<tr>
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<td>NS</td>
<td>(n = 4)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
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<td>(n = 3)</td>
</tr>
</tbody>
</table>

Densitometric quantitation of autoradiographs of Northern blots for CaM mRNA is reported with average values ± SE. Data are normalized to control (100%).
teins and, hence, rapidly increases O-GlcNAc levels of the cells. Although O-GlcNAcase is expressed in all tissues, it is localized predominantly to the cytoplasm (4). Glucagon weakly stimulates Sp1 synthesis but does not glycosylate it, and the unglycosylated Sp1 remains localized to the cytoplasm. When STZ was added to cells treated with glucagon, there was enhanced glycosylation of Sp1 with a concomitant increase in CaM gene transcription, similar to insulin. McClain et al. (12) have suggested that enhanced glycosylation of Sp1 with a concomitant increase in CaM gene transcription, similar to insulin. McClain et al. (12) have suggested that O-GlcNAc transferase (OGT), an enzyme that glycosylates proteins, plays a central role in the insulin-signaling cascades. In addition to supporting this concept, we suggest that both OGT and O-GlcNAcase enzymes are likely to be involved in insulin signaling. Thus these enzymes probably play a more critical role than the size of the intracellular UDP-GlcNAc pool in regulating O-glycosylation of Sp1. The ability of STZ, an inhibitor of O-GlcNAcase, to stimulate CaM mRNA accumulation in cells treated with either insulin or glucagon lends excellent support for such a proposal.

In summary, CaM gene expression is stimulated by insulin, with a combination of individual events contributing, i.e., enhanced Sp1 protein synthesis, O-glycosylation, and nuclear localization. Glucagon, which generally antagonizes insulin although promoting net Sp1 synthesis, neither O-glycosylates Sp1 nor promotes its movement to the nucleus and does not enhance CaM gene transcription. Although it would be nice to generalize our findings, the CaM gene is unique and may undergo regulatory changes that are like/unlike other genes.

**DISCLOSURES**

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