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Reduced O Glycosylation of Sp1 Is Associated with Increased Proteasome Susceptibility

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Sp1 is a ubiquitously expressed transcription factor that is particularly important for the regulation of TATA-less genes that encode housekeeping proteins. Most growth factors and receptors are also encoded by such genes. Sp1 is multiply O glycosylated by covalent linkage of the monosaccharide *N*-acetylglucosamine (*O*-GlcNAc) to serine and threonine residues. Based on an earlier observation that growth factor gene transcription can be regulated by glucose and glucosamine in vascular smooth muscle cells, we determined whether Sp1 glycosylation could be regulated and if this modification altered Sp1 function. We found that Sp1 becomes hyperglycosylated when cells are exposed to 5 mM glucosamine, whereas under glucose starvation, stimulation with cyclic AMP (cAMP) results in nearly complete deglycosylation of this protein. Correlating with this hypoglycosylated state, Sp1 is rapidly proteolytically degraded by an enzyme(s) that can be inhibited by specific proteasome inhibitors, lactacystin and LLnL. Treatment of cells with glucose or glucosamine protects Sp1 from cAMP-mediated degradation, whereas blockade of glucosamine synthesis abrogates glucose but not glucosamine protection. This effect on Sp1 is specific, in that the Stat-3 and E2F transcription factors did not undergo degradation under these conditions. The *O*-GlcNAc modification of Sp1 may play a role as a nutritional checkpoint. In the absence of adequate nutrition, Sp1 becomes hypoglycosylated and thereby subject to proteasome degradation. This process could potentially result in reduced general transcription, thereby conserving nutrients.

In all organisms, cell growth is ultimately coupled to the availability of nutrients. This substrate availability can alter a variety of cellular processes, thereby controlling macromolecular synthesis and mitosis. For example, in *Saccharomyces cerevisiae*, phosphate deprivation can alter the activity of cyclin-dependent protein kinases, resulting in the arrest of the cell cycle (33, 43). Similarly, the availability of a carbon source, such as glucose, can alter the progress of yeast through the cell cycle (3). In multicellular organisms, much of this nutrient regulation is obviated, because the concentrations of these substrates are maintained by homeostatic mechanisms. In vertebrates, the cell is exposed to nearly constant concentrations of glucose which are maintained in the animal through hormonal controls. Nevertheless, marked deviations of this pattern of glucose homeostasis do occur with the development of the disease diabetes mellitus. In its uncontrolled form, diabetes is characterized by the exposure of cells to high glucose concentrations, leading to chronic complications (15a) that include proliferative changes in the blood vessel wall (40). This proliferative effect of high glucose concentrations has recently been postulated to result from glucose-induced changes in vascular cell growth (23), perhaps as a result of altered gene regulation. Our laboratory has shown that the transcription of growth factor genes can be altered by changing the extracellular glucose concentration (32).

The mechanisms by which glucose alters gene expression remain unknown. For its effect on growth factor gene transcription, glucose must be metabolized to glucosamine (39, 44). Glucosamine also mimics the effect of glucose on growth factor gene expression (12). While glucose can enter a myriad of metabolic pathways, glucosamine is used primarily as a substrate for protein glycosylation, suggesting that the effect of glucose on growth factor gene transcription may be mediated through altered glycosylation of cellular proteins. Of particular interest with regard to gene transcription is the observation that many transcription factors are posttranslationally modified by O glycosylation (8, 25, 30, 36). This modification involves the covalent linkage of the monosaccharide *O*-GlcNAc to serine or threonine residues in these proteins. The first transcription factor known to contain this modification was Sp1 (25). Interestingly, Sp1 is a ubiquitous transcription factor that recognizes a GC box sequence present in many, if not most, cellular and viral promoters (17, 27, 28). The activity of Sp1 is generally believed to be constitutive, although there is some evidence that its activity can be modulated (2, 4, 7, 41, 42, 46). The modulation of Sp1 activity would be expected to have a generalized effect on gene transcription, but this effect would be most marked in those genes containing GC-rich promoters and no TATA box. Promoters with such characteristics are typical of housekeeping genes, whose transcription is highly dependent on Sp1 (35). The studies described here were designed to determine if the glycosylation state of Sp1 can be modulated by extracellular signals, including glucose deprivation, and if so, whether the glycosylation state could modify the properties of this transcription factor. We found that glucose deprivation combined with adenylate cyclase activation resulted in the generation of hypoglycosylated Sp1. While protein hypoglycosylation may have been general under these experimental conditions, we found that Sp1 was specifically and markedly more susceptible to proteolytic degradation by a protease having inhibitor characteristics of the proteasome. While the experimental means we used for inducing hypoglycosylation may be artificial, this observation suggests that the glycosylation state of either Sp1 or some other nuclear protein may determine the stability of Sp1 in the cell. Whether Sp1 hypoglycosylation and proteolytic degradation represent part

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of a physiological response to nutritional deprivation remains to be seen, but these results do indicate that Sp1 undergoes a profound change in its properties in response to the *O*-GlcNAc modification.

MATERIALS AND METHODS

Chemicals. E64-D (2*S*,3*S*-*t*-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester) and LLnL (*N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal) were purchased from Sigma Chemical (St. Louis, Mo.). Lactacystin was obtained from E. J. Corey (Harvard University, Boston, Mass.) (19). These drugs were dissolved in dimethyl sulfoxide (DMSO) before use, and throughout the experiment, the final concentration of DMSO in cell culture, including control cultures, was kept at 0.5%.

Cell culture. NRK cells (38) and MDA468 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) (GIBCO/BRL, Grand Island, N.Y.), 100 μg of penicillin (Sigma Chemical) per ml, and 50 μg of gentamicin (Sigma Chemical) per ml at 37°C in a humidified incubator with 7.5% CO_2. For stimulation assays, exponentially growing cells were seeded at 5×10^5 cells per 60-mm-diameter dish at approximately 40% confluency. After overnight incubation, the medium was changed to glucose-free DMEM containing 10% FCS, and the cells were incubated for an additional 15 h. When indicated, the cells were then treated with combinations of 100 μM forskolin (Sigma Chemical) and 5 mM glucosamine, usually in glucose-free DMEM (unless otherwise stated) containing 10% FCS, and incubation was continued for an additional 24 h. Lactacystin, LLnL, and E64-D were added at a concentration of 20 μM 5 h prior to harvest.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA). Nuclear extract was prepared according to the method of Dignam et al. (16) and had a protein concentration of 6.2 mg/ml, as estimated by the Bradford assay with bovine serum albumin as a standard. The double-stranded DNA oligonucleotide probes for the gel shift experiments contained the consensus Sp1 or E2F binding site (Promega) labeled by polynucleotide kinase (New England Biolabs). Radiolabeled double-stranded oligonucleotides were desalted through a Sephadex G-25 spin column. Typically, the specific activities of oligonucleotide probes were 10^5 cpm/ng of DNA. The probes were stored at −20°C until use. Binding reactions were performed in a volume of 30 μl in 50 mM KCl, 12.5 mM HEPES (pH 7.6), 6.25 mM $MgCl_2$, 0.05 mM EDTA, 0.05% Nonidet P-40, 0.5 mM dithiothreitol (DTT), and 5% glycerol and were incubated for 30 min on ice. The reaction mixtures contained 2 μg of poly(dI-dC) (Pharmacia LKB Biotechnology, Piscataway, N.J.) and 0.15 ng of labeled DNA. For the competition experiments, 7.5 ng of unlabeled oligonucleotide was added to the reaction mixture. Rabbit polyclonal anti-Sp1 (Santa Cruz Biotechnology, Santa Cruz, Calif.) and rabbit polyclonal anti-AP2 (Santa Cruz Biotechnology) antibodies were added to the extract prior to the addition of the probe for the supershift assay. The samples were loaded on a 5% polyacrylamide gel (acrylamide-bisacrylamide, 30:1) containing 0.5× Tris-borate-EDTA buffer plus 2% glycerol, and the gel was electrophoresed at 200 V at room temperature for approximately 1.5 h. The gels were then dried and exposed to X-ray film with an intensifying screen at −80°C.

Immunoprecipitation and immunoblotting. The polyclonal antiserum to Sp1 (3517) was raised against the C-terminal portion of Sp1 that had been expressed in *Escherichia coli*, as previously described (46). The Sp1 antibody was affinity purified (46) by incubating the antiserum with Sp1 protein immobilized on a nitrocellulose membrane followed by low pH elution of the antibody. The monoclonal antibody RL2, specific for the GlcNAc moiety on proteins, was a gift from L. Gerace (Scripps Clinic, La Jolla, Calif.). The Stat-3 antibody was purchased from Transduction Laboratories, Lexington, Ky.

Immunoprecipitations were performed according to standard procedures (22). Cells were scraped from the cell culture plates, pelleted, and washed twice with cold phosphate-buffered saline. The cell pellets were resuspended in ice-cold 20 mM HEPES (pH 7.9), 500 mM NaCl, 20% glycerol, 1 mM DTT, 0.1% Nonidet P-40, and 1 mM phenylmethanesulfonyl fluoride. The cells were gently vortexed intermittently over a period of 30 min. Cellular debris was pelleted for 30 min at 135,000 × *g* at 4°C in a TLA100.3 rotor (Beckman, Palo Alto, Calif.). The resulting high-salt supernatants were the source of cellular glycoproteins containing monosaccharide *O*-GlcNAc modification. The supernatants were dialyzed against binding buffer (20 mM HEPES–KOH at pH 7.9, 0.25 M KCl, 2 mM $MgCl_2$, 10 μM $ZnSO_4$, 1 mM DTT, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride). The protein concentration was determined by using the Bio-Rad D_c Protein Assay (Bio-Rad Laboratories, Hercules, Calif.). For each immunoprecipitation reaction, approximately 500 μg of cell extract was incubated with 4 μg of affinity-purified antibodies in binding buffer at 4°C for several hours. Protein A-Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, N.J.) was then added, and the mixture was incubated at 4°C on a rotating wheel for 2 h. The antibody-protein A complexes were pelleted by centrifugation (1,000 × *g*) and washed four times with binding buffer. The pellets were resuspended in 1× sodium dodecyl sulfate (SDS) protein sample buffer, boiled, and analyzed by SDS–7.5% polyacrylamide gel electrophoresis (PAGE). The protein was transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) for Western blot analysis. The immunoblots were developed with a 1:1,000 dilution of affinity-purified antibodies, and the signals were detected with the Enhanced Chemiluminescence System (Amersham, Arlington Heights, Ill.).

Vaccinia virus expression and purification of recombinant Sp1. The full-length human Sp1 cDNA (kindly provided by James Kadonaga) was cloned into vector pTM3, and a recombinant vaccinia virus was generated by selection methods previously described (34). Recombinant Sp1 was overexpressed after coinfection with Sp1 cDNA containing recombinant vaccinia viruses and vTF7-3 viruses. Recombinant Sp1 was purified as previously described (5).

RESULTS

Inhibition of DNA-binding activity of Sp1 in forskolin-treated NRK cells. MDA468 cells and NRK cells were deprived of glucose for 15 h and then treated for 24 h without (Fig. 1A, lanes 2 and 5) or with (Fig. 1A, lanes 3 and 6) forskolin, an agent that increases intracellular cyclic AMP (cAMP) production by direct activation of adenylate cyclase in the plasma membrane (45). Nuclear extracts from these cells were tested for the presence of Sp1 DNA-binding activity by using an EMSA with an oligonucleotide containing a consensus Sp1 binding site. This treatment with forskolin under glucose starvation conditions resulted in the virtual loss of Sp1 DNA-binding activity in the NRK (Fig. 1A, lane 6) but not the MDA468 (Fig. 1A, lane 3) cells. Exposure of these cells to 5 mM glucosamine prior to the treatment with forskolin completely prevented this loss of Sp1 activity in the NRK cells (Fig. 1A, lane 7). This result implies that glucosamine abolishes the forskolin effect for Sp1 DNA-binding activity. The result also implies that this effect is cell specific. The gel shifts observed, using the GC box Sp1 binding site, were attributable to Sp1. Purified recombinant Sp1 shifted the probe identically to the protein in the NRK and MDA468 cell nuclear extracts (Fig. 1A, lane 10). The NRK gel shift could be competed with a 50-fold excess of Sp1 GC box (Fig. 1B, lane 2) but not with the GC-rich E2F binding site (Fig. 1B, lane 3). The Sp1-DNA complex could be supershifted with an Sp1 antibody (Fig. 1B, lanes 4 and 5) but not with an antibody to AP2. AP2 is a transcription factor that also recognizes a GC-rich element (Fig. 1B, lane 6).

Chen et al. have shown that CV-1 nuclear extracts contain a negative regulator(s) of Sp1 (Sp1-I), which inhibits formation of Sp1-DNA complexes, as determined by EMSA (7). To determine whether forskolin could activate a factor that inhibits Sp1 DNA-binding activity, an experiment was performed in which the nuclear extracts were mixed. We incubated a nuclear extract of NRK cells which had been glucose starved and then forskolin treated with a nuclear extract of NRK cells that had been glucose starved but not forskolin treated (Fig. 1A, lane 8). The mixing of these extracts resulted in a marked reduction in the DNA-binding activity of Sp1. However, if nuclear extract from cells pretreated with glucosamine plus forskolin was mixed with the extract from glucose-starved cells, there was no loss of Sp1 DNA-binding activity (Fig. 1A, lane 9). These results suggest that the nuclear extracts from forskolin-treated, glucose-deprived cells contain an activity that obliterates the DNA-binding activity of Sp1. To determine the effect of mixing the extracts on the quantity of Sp1 protein, Western blot analysis was performed following the mixing of the extracts (Fig. 1C, lanes 1 to 5). The Western blotting was performed with an antibody that was raised against a bacterially expressed fragment of Sp1 (46). Addition of increasing amounts of nuclear extract from NRK cells that had been glucose starved and forskolin treated to the extract of cells that had only been glucose starved resulted in a dose-dependent depletion of Sp1 protein. In contrast, when the extract from cells treated with both glucosamine and forskolin was added to the extract from cells treated with 5 mM glucosamine, no loss of Sp1 protein was detected (Fig. 1C, lanes 6 and 7).

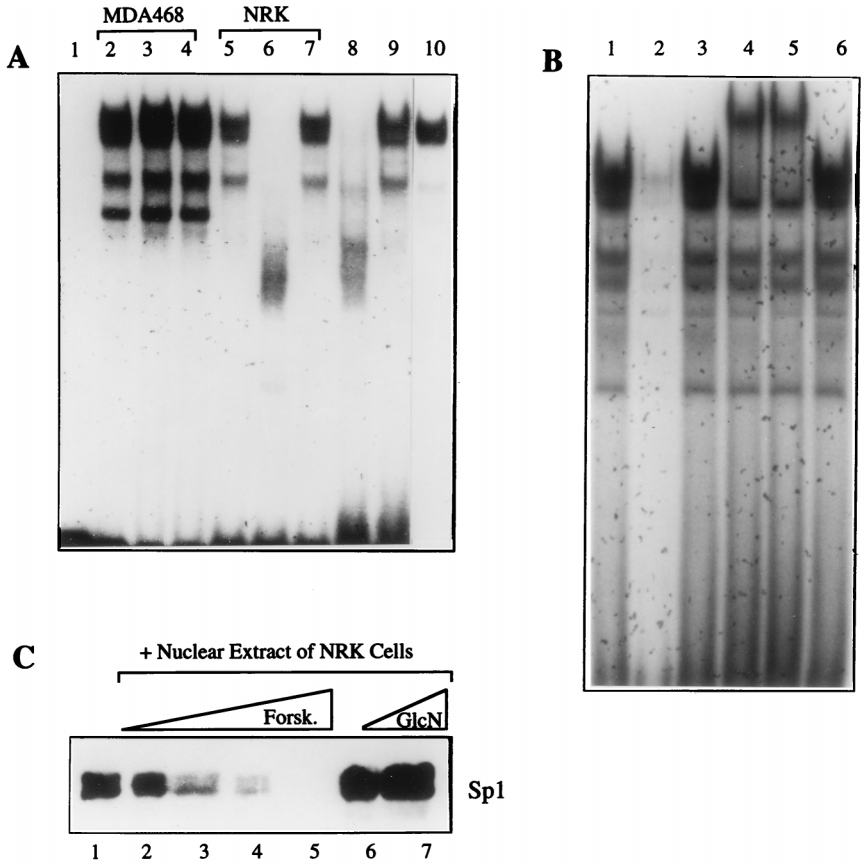

FIG. 1. Cell-type-specific inhibition of the DNA-binding activity of Sp1 in nuclear extracts of forskolin-treated cells. (A) Sp1 DNA-binding activity in the indicated cell types was determined by EMSA. The ^{32}P-labeled oligonucleotide (0.15 ng) containing the consensus Sp1 binding site with 2 μg of poly(dI-dC) was used for binding assays with nuclear extract containing 8 μg of protein. Lane 1, no nuclear extract; lanes 2 and 5, nuclear extracts from cells that had been glucose starved; lanes 3 and 6, nuclear extracts from glucose-starved cells treated with 100 μM forskolin; lanes 4 and 7, nuclear extracts from glucose-starved cells treated with 5 mM glucosamine. Lane 8 contains a mixture of equal amounts (8 μg each) of nuclear extracts from untreated (glucose-starved) NRK cells and NRK cells which were glucose starved and forskolin treated. Lane 9 contains a mixture of nuclear extracts from untreated NRK cells and NRK cells treated with forskolin plus glucosamine after glucose starvation. Lane 10, 5 ng of vaccinia virus-expressed recombinant Sp1. The NRK and MDA468 cells were incubated in glucose-free DMEM for 15 h before treatment with forskolin and/or glucosamine. (B) EMSA was performed to identify the shifted bands. Each reaction mixture contained 8 μg of nuclear extract from NRK cells treated with glucose-free DMEM for 15 h and the ^{32}P-labeled Sp1 binding site probe. Lane 1, nuclear extract alone; lane 2, 50-fold excess (7.5 ng) of unlabeled Sp1 consensus site oligonucleotide; lane 3, 50-fold excess (7.5 ng) of unlabeled E2F binding site oligonucleotide; lanes 4 and 5, 0.5 and 1 μg of Sp1 antibody, respectively; lane 6, 1 μg of AP2 antibody. (C) The quantity of Sp1 protein in the nuclear extracts was determined by Western blot analysis with a polyclonal Sp1 antibody. Nuclear extract (40 μg) from glucose-starved NRK cells (Fig. 2B, lane 1) was mixed with 10, 20, 40, and 80 μg (lanes 2, 3, 4, and 5, respectively) of nuclear extract from forskolin-treated, glucose-starved NRK cells. Nuclear extract (40 μg) from glucose-starved cells was mixed with 20 (lane 6) or 80 μg (lane 7) of extract from cells treated with both glucosamine and forskolin. The mixtures were incubated on ice for 30 min and separated by SDS–7.5% PAGE. The blot was probed with an Sp1 rabbit antiserum.

Glucose starvation is necessary for forskolin effect on the DNA-binding activity of Sp1. The experiments discussed above were performed on NRK cells that had been deprived of glucose for 15 h prior to forskolin treatment. To determine the necessity of this glucose starvation, the effects of forskolin on Sp1 were compared for cells that were glucose starved and cells that were grown in 5 mM glucose. While we reproducibly observed a loss of Sp1 DNA-binding activity in cells treated with forskolin under glucose starvation conditions (Fig. 2A, lane 2), the presence of glucose in the medium blocked this effect of forskolin (Fig. 2A, lanes 5 and 6). Glucosamine at 5 mM also blocked the forskolin effect (Fig. 2A, lanes 3 and 4). This observation indicates that glucose deprivation is necessary for the observed effect of forskolin on Sp1. Furthermore, both glucose and glucosamine block this forskolin effect. The effect of these treatments on Sp1 appear to be relatively specific. The same nuclear extracts were tested for the DNA-binding activity of another transcription factor, E2F, with a labeled oligonucleotide recognized by this transcription factor. The sugar and forskolin treatments that altered Sp1 DNA-binding activity had no significant effect on E2F DNA-binding activity (Fig. 2B), suggesting some degree of specificity. This result also suggests that the nuclear extracts were not subject to general protein degradation.

The major cause of this loss of Sp1 DNA-binding activity was either of the following: (i) an Sp1 inhibitory factor (Sp1-I) (7) could be activated under the conditions of forskolin treatment, resulting in inhibition of the DNA-binding activity of Sp1 or (ii) forskolin treatment could result in changes in the quantities of Sp1 in the nuclear extracts. To distinguish between these alternatives, the quantity of Sp1 protein was determined by Western blot analysis. As shown previously in Fig. 1C and now in Fig. 2C, the amount of Sp1 protein was markedly diminished upon forskolin treatment of glucose-starved cells. Congruent with the EMSA result, glucose or glucosamine prevented this forskolin-induced loss of Sp1 protein. Again, this effect on Sp1 appeared to be relatively specific, in that the same Western blot, when probed for the transcription factor,

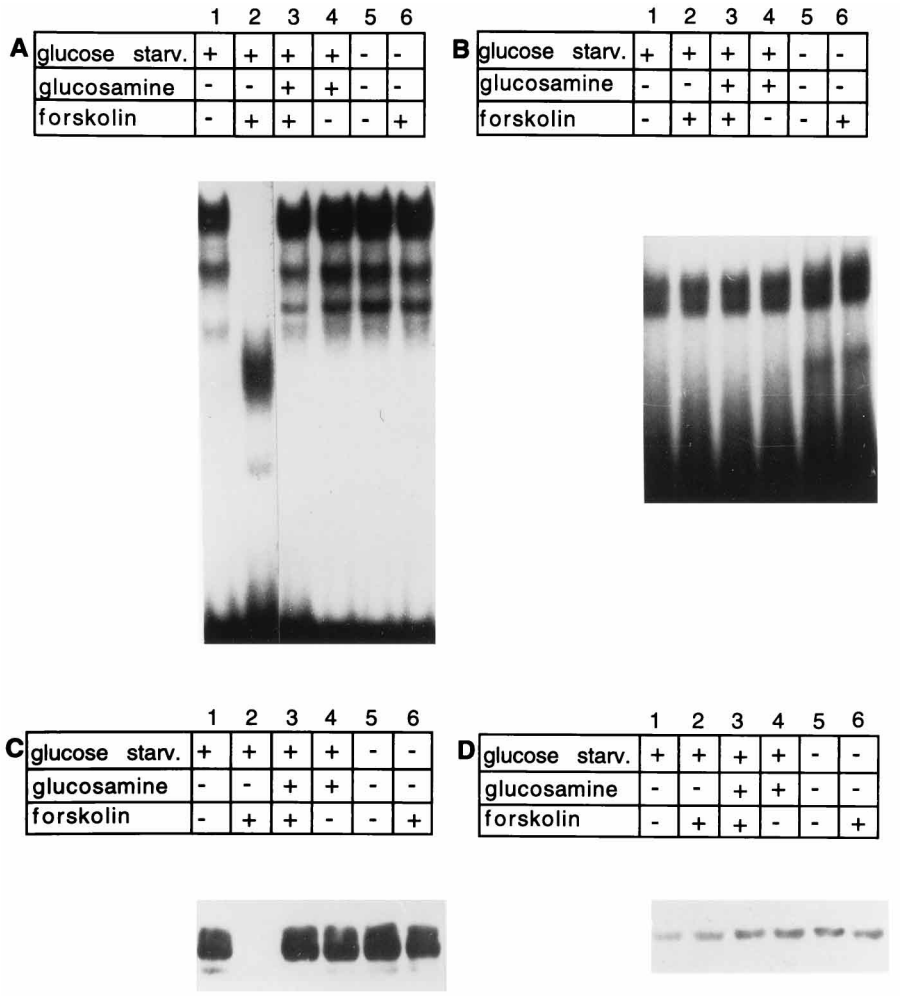

FIG. 2. Glucose starvation is necessary for the effect of forskolin on the Sp1 DNA-binding activity and protein level. EMSA or Western blot analysis was performed with nuclear extracts from NRK cells treated as indicated with 15 h of glucose starvation, 5 mM glucosamine, and 100 μM forskolin. (A) EMSA was performed with 8 μg of nuclear extract protein from cells treated as indicated, using the ^{32}P-labeled consensus Sp1 binding site oligonucleotide (0.5 pmol). (B) EMSA was performed with the same extracts, using the consensus E2F binding oligonucleotide as a probe. (C) Western blot analysis of the same nuclear extracts (40 μg of protein) was performed with polyclonal Sp1 antibody. The nuclear proteins were separated by SDS-PAGE (7.5% polyacrylamide) and then immunoblotted with Sp1 antibody. (D) The Western blot described above for panel C was stripped and probed with an antibody directed at the transcription factor Stat-3.

Stat-3 (Fig. 2D), showed no loss of Stat-3 under the conditions that resulted in a loss of Sp1.

Blockade of glucosamine synthesis mimics the effect of glucose starvation. 6-Diazo-5-oxonorleucine (DON) is an inhibitor of the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT), which is rate limiting for hexosamine synthesis. DON is a glutamine analog that covalently binds to GFAT (3, 8, 44), thereby permanently inhibiting this enzyme. Treatment of cells with this GFAT inhibitor results in the hypoglycosylation of many cytoplasmic and nuclear proteins (39, 44). The observation that both glucose and glucosamine are sufficient in blocking the forskolin effect on Sp1 raised the question as to whether metabolism of glucose to glucosamine is necessary for this action of glucose. We therefore used the GFAT inhibitor to block metabolism of glucose to glucosamine to probe this question. NRK cells were grown in 5 mM glucose and treated with or without the GFAT inhibitor (40 μM DON) prior to exposure to forskolin. As before, forskolin had no effect on Sp1 DNA-binding activity (Fig. 3A, lane 2) or the quantity of Sp1 protein in the nuclear extracts (Fig. 3B, lane 2) when added to the cells in the presence of 5 mM glucose. However, when the cells were treated with DON in the presence of glucose, forskolin treatment resulted in the loss of Sp1 activity and protein (Fig. 3, lanes 4). Glucosamine, which enters the hexosamine pathway downstream of GFAT, blocked the effect of forskolin (Fig. 3, lanes 6 and 8). These treatments with sugars and forskolin had no significant effects on the amount of Stat-3 in the nuclear extracts (Fig. 3B, lower panel). This result implies that synthesis of glucosamine from glucose is required to block the effect of forskolin on Sp1.

Time course of forskolin effect on total Sp1 protein level in NRK cells. The absence of Sp1 activity and protein in nuclear extracts could have resulted from a change in the subcellular localization of this transcription factor in response to forskolin treatment. To address this question, the amount of Sp1 protein was measured by Western blotting in a total NRK cell extract made in SDS-PAGE sample buffer. At the same time, a time course study of forskolin action was performed (Fig. 4). Following an overnight glucose starvation, cells were treated for the indicated times with forskolin or vehicle (DMSO), and the amount of Sp1 protein in the whole-cell extracts was examined by Western blotting. While 3 h of forskolin treatment had no

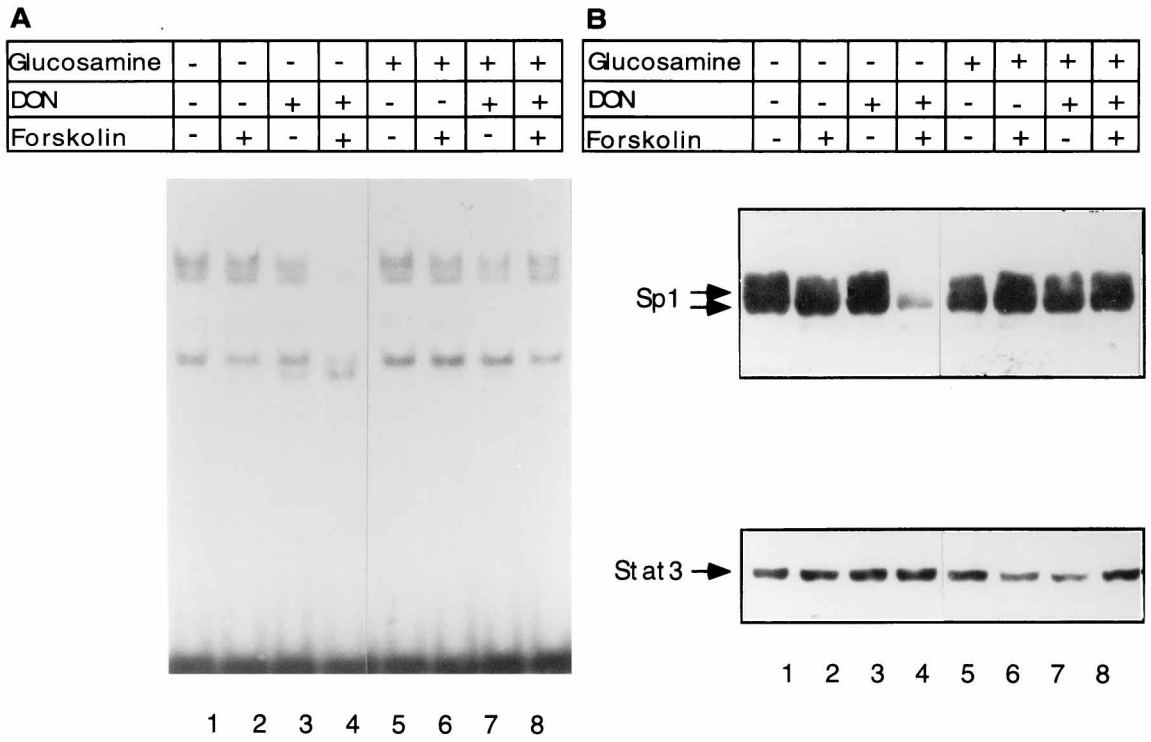

FIG. 3. Blockade of glucosamine synthesis is permissive for the forskolin effect on Sp1. (A) NRK cells were treated as indicated with combinations of 40 μM DON, 5 mM glucosamine, and 100 μM forskolin. DON and/or glucosamine was added to the cells 15 h prior to the addition of forskolin. Nuclear extracts (8 μg of protein) were analyzed for Sp1 DNA-binding activity by EMSA. (B) The quantity of either Sp1 or Stat-3 in the same nuclear extracts (40 μg) was determined by Western blot analysis with either polyclonal Sp1 antibody (upper panel) or monoclonal Stat-3 antibody (lower panel).

significant effect on the quantity of Sp1 in the cells, treatment for 6 h through 48 h resulted in a virtually complete loss of the protein. The blot was stripped and reprobed for Stat-3. The quantity of this transcription factor did not change appreciably with these treatments. This finding suggests that forskolin treatment of glucose-starved cells results in a marked and relatively rapid loss of Sp1 protein in NRK cells. This effect on Sp1 appears to apply to all of the intracellular pools in which Sp1 might reside.

Effect of protease inhibitors on forskolin-induced loss of Sp1. The forskolin-induced loss of Sp1 protein in the glucose-starved NRK cells occurs rapidly. This loss could result from the cessation of Sp1 synthesis or the acceleration of Sp1 degradation. In either case, the rapid loss of Sp1 protein indicates that Sp1 has a relatively short half-life, particularly after forskolin treatment. This observation, in conjunction with the observation that mixing extracts from forskolin-treated and untreated cells results in the loss of Sp1 DNA-binding activity, suggests that this loss of Sp1 results from proteolytic degradation. Two major proteolytic systems have been described for eukaryotic cells (10): the lysosomal system and the proteasome. Lysosomal (or, in yeast, vacuolar) proteases are principally involved in the degradation of proteins derived from the plasma membrane or proteins of exogenous origin imported through the endocytic apparatus. Most short-lived cytoplasmic and nuclear proteins, however, are degraded by the 26S proteasome complex. This cytoplasmic and nuclear organelle contains a multicatalytic 20S core and usually requires prior covalent attachment of multiple ubiquitin chains to the substrate (10, 21). The proteasome is highly substrate specific as a result of both substrate labeling with ubiquitin and the structural requirement for energy-dependent protein denaturation prior to the proteolysis.

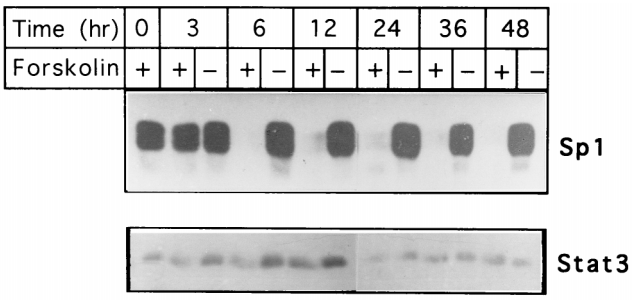

FIG. 4. Time course of the forskolin effect on the Sp1 protein level in NRK cells. NRK cells were glucose starved for 15 h and then stimulated with 100 μM forskolin for various times as indicated. Total cellular proteins were extracted with SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE (7.5% polyacrylamide). The separated proteins were analyzed by immunoblotting with polyclonal Sp1 (upper panel) or monoclonal Stat-3 (lower panel) antibodies.

The selective and rapid loss of Sp1 following forskolin treatment prompted us to investigate whether the proteasome system was involved. We made use of proteasome inhibitors to determine whether this loss of Sp1 could be blocked. The peptide aldehyde lactacystin, derived from *Streptomyces*, is a potent irreversible inhibitor of the trypsin-like and chymotrypsin-like peptidyl-glutamyl peptide-hydrolyzing activities of the 20S proteasome (19). Lactacystin has not been found to inhibit any other of the known proteases. Addition of lactacystin to NRK cells for 5 h prior to harvest markedly inhibited the

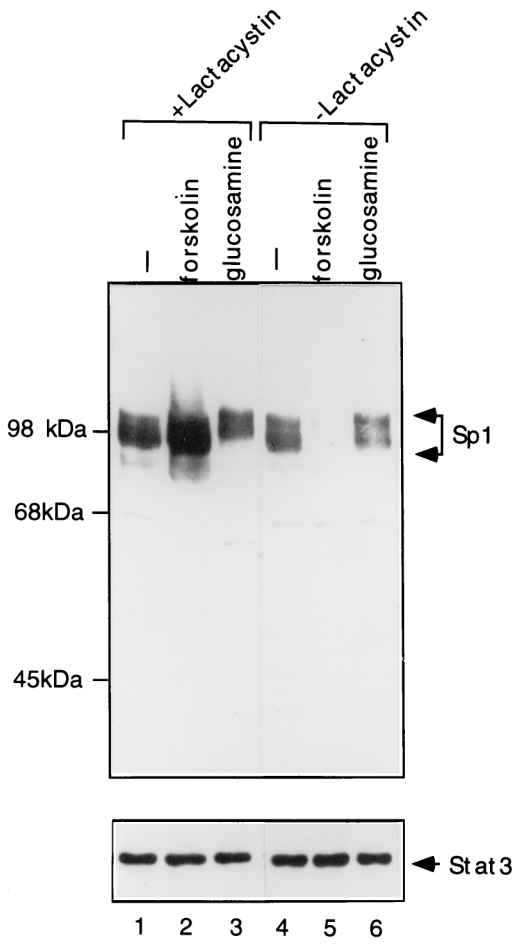

FIG. 5. Lactacystin, an inhibitor of the 26S proteasome, blocks the forskolin-induced degradation of Sp1. NRK cells were glucose starved for 15 h and then treated as indicated with either 100 μM forskolin (lanes 2 and 5) or 5 mM glucosamine (lanes 3 and 6) for 24 h. The proteasome inhibitor lactacystin was added to the cells for 5 h prior to harvest at a concentration of 20 μM. Whole-cell lysates in SDS sample buffer were separated by SDS-PAGE (7.5% polyacrylamide) and analyzed by Western blotting with polyclonal Sp1 (upper panel) or monoclonal Stat-3 (lower panel) antibodies.

forskolin-induced loss of Sp1 protein (Fig. 5). This result suggests that forskolin treatment results in the proteolytic degradation of Sp1, thereby leading to its loss from the cell extracts. That lactacystin blocks this loss is compatible with proteasome involvement in Sp1 degradation. Of note, lactacystin pretreatment did not result in the accumulation of higher-molecular-weight forms of Sp1, which might have been attributed to polyubiquitination. However, this result alone does not rule out Sp1 ubiquitination, in that Sp1 is a relatively large protein and a small amount of ubiquitin would not significantly alter the mobility of Sp1 in SDS-PAGE. Other proteins (c-Jun [26] and ornithine decarboxylase [18]) have been shown to be subject to proteasome degradation in the absence of ubiquitination.

The dose-dependent effects of protease inhibitors on steady-state levels of Sp1 in NRK cells were assessed by immunoblot analysis (data not shown). Lactacystin was half-maximally effective at approximately 10 μM and maximally effective at 20 μM at blocking the forskolin effect on Sp1. *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL), another potent inhibitor of the 20S proteasome, blocked the forskolin effect, whereas the calpain inhibitor, E64-D, had no effect on the forskolin-induced degradation of Sp1.

Effects of forskolin and glucosamine on level of *O*-GlcNAc in Sp1. Glucose or glucosamine treatment of NRK cells blocks the effect of forskolin on Sp1 degradation, whereas inhibition of glucosamine synthesis or glucose deprivation is permissive for this forskolin effect. Since Sp1 is posttranslationally modified by the addition of *O*-GlcNAc residues, we determined the effect of forskolin and glucosamine treatment of NRK cells on Sp1 O glycosylation in an attempt to correlate this modification with the stability of Sp1. In order to observe the glycosylation state of Sp1, the cells were treated with lactacystin to prevent the loss of Sp1 protein as a result of the forskolin treatment. Following treatment of the glucose-starved cells (Fig. 6A, lane 1) with either glucosamine (Fig. 6A, lane 2) or forskolin (Fig. 6A, lane 3), Sp1 was immunoprecipitated and analyzed by Western blotting. As expected, lactacystin prevented the loss of Sp1 protein following forskolin treatment. However, when the same blot was probed with the GlcNAc-specific monoclonal antibody RL-2 (39, 44, 47), forskolin treatment resulted in the complete loss of the RL-2 epitope (GlcNAc) on Sp1 (Fig. 6A, lane 3). Glucosamine treatment resulted in the appearance of apparently higher-molecular-weight glycosylated forms of Sp1. In addition, the O-glycosylated nuclear pore protein (p62) (31) that we have shown to coprecipitate with Sp1 (20) undergoes changes in O glycosylation in parallel to Sp1 (data not shown), suggesting a more general effect on O glycosylation of forskolin and glucosamine. Treatment of the cells with forskolin in the presence of glucosamine (Fig. 6A, lane 4) or glucose (Fig. 6A, lane 5) does not result in the loss of the RL-2 signal. Interestingly, these changes in the level of Sp1 glycosylation were not observed in MDA468 cells (Fig. 6B). In these cells, neither forskolin nor glucosamine treatment resulted in a change in Sp1 *O*-GlcNAc modification. Treatment of these cells with glucose starvation and forskolin also did not result in a loss of Sp1 DNA-binding activity (Fig. 1), thus sustaining the correlation between Sp1 loss and Sp1 hypoglycosylation.

Mixing an extract from glucose-starved cells with an extract from glucose-starved cells treated with forskolin results in the degradation of all Sp1 protein, including the glycosylated Sp1 derived from cells that had not been exposed to forskolin (Fig. 1C). To determine the effect of mixing these extracts on Sp1 glycosylation, we included LLnL in the incubation to prevent this degradation. As shown in Fig. 6A, glucose starvation of the cells followed by incubation of the extract for 30 min did not result in deglycosylation of Sp1 (Fig. 6C, lane 1), whereas the forskolin treatment with glucose starvation did (Fig. 6C, lane 2). Mixing of these two extracts and incubation for 30 min resulted in the partial deglycosylation of the Sp1 protein derived from the extract of the cells not treated with forskolin. Of interest, other unidentified proteins detected with the RL-2 antibody also became less glycosylated following exposure to the forskolin extract. This result suggests that the forskolin extract contains an *O*-GlcNAc-specific *N*-acetylglucosaminidase (*O*-GlcNAcase) with a relatively greater net activity than the enzyme in the extract from the glucose-starved untreated cells. This *O*-GlcNAcase does not appear to be substrate specific. Taken together with the result shown in Fig. 1C, i.e., that the mixture of equal quantities of these extracts results in the partial degradation of Sp1, it would appear that the forskolin-induced deglycosylation of either Sp1 or some other protein is a prerequisite for the proteasome degradation of Sp1.

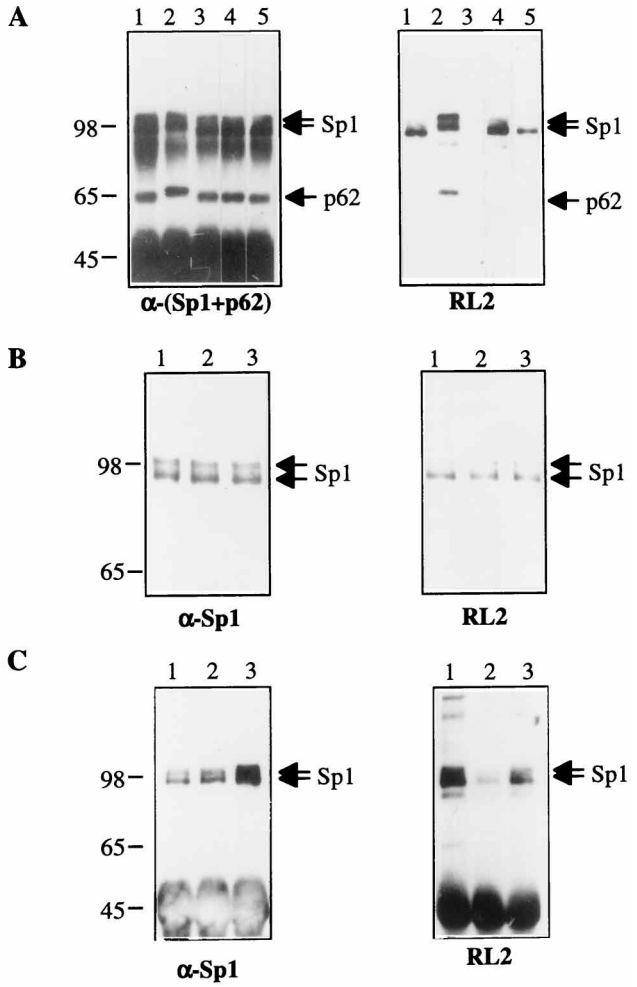

FIG. 6. The forskolin- or glucosamine-induced changes in *O*-GlcNAc content of Sp1. (A) NRK cells were glucose starved for 15 h (lane 1) and then treated with 5 mM glucosamine (lane 2), 100 μM forskolin (lane 3), both 100 μM forskolin and 5 mM glucosamine (lane 4), or both 100 μM forskolin and 5 mM glucose (lane 5) for 24 h. The cells were then incubated for 5 h at 37°C with 20 μM lactacystin. After incubation, cellular proteins were extracted, and the Sp1 protein was immunoprecipitated and analyzed by SDS-PAGE and Western blotting with the indicated antibodies. The membrane was probed with the mixture of polyclonal Sp1 (3517) and nuclear pore protein p62 antibodies (left panel). The membrane was stripped and reprobed with monoclonal RL2 antibody (right panel). (B) MDA468 cells were glucose starved for 15 h (lane 1) and then treated with either 5 mM glucosamine (lane 2) or 100 μM forskolin (lane 3) in glucose-free DMEM for 24 h. The cells were then incubated for 5 h at 37°C with 20 μM lactacystin. After incubation, cellular proteins were extracted, and the Sp1 protein was immunoprecipitated and analyzed by SDS-PAGE and Western blotting with the indicated antibodies. The membrane was probed with the polyclonal Sp1 antibody (3517) (left panel). The membrane was stripped and reprobed with the monoclonal RL2 antibody (right panel). (C) NRK cells were glucose starved for 15 h and then treated without (lane 1) or with (lane 2) 100 μM forskolin in glucose-free DMEM for 24 h. The cells were then incubated for 5 h at 37°C with 20 μM lactacystin. After incubation, cellular proteins were extracted and incubated on ice for 30 min. A 1:1 mixture of the extracts shown in lanes 1 and 2 was incubated on ice for 30 min (lane 3). All incubation mixtures contained 20 μM lactacystin. The Sp1 protein was immunoprecipitated and analyzed by SDS-PAGE and Western blotting with either polyclonal Sp1 (3517) (left panel) or RL2 monoclonal (right panel) antibodies.

DISCUSSION

The transcription factor Sp1 plays a central role in the transcriptional regulation of those genes whose promoters are GC rich and lack a TATA box. Indeed, transcription from such promoters is highly dependent on Sp1 (35). Most of these promoters are commonly associated with genes that encode ubiquitous proteins that are often thought to be constitutively expressed. However, several of these Sp1 target genes encode enzymes involved in DNA synthesis, growth factors, protein kinases, receptors, and other regulatory proteins. In some cases, the expression of such genes has been shown to be regulated, often in association with growth stimulation and progression through the cell cycle. While in some of these genes, this cell cycle modulation has been largely attributed to the regulation of E2F activity by the phosphorylation of the retinoblastoma (Rb) protein (48), there has been some evidence that Sp1 may also contribute to this regulation (2, 4, 7, 41, 42, 46). In this paper we demonstrate that, under albeit experimental circumstances, Sp1 is a very labile protein. To observe this lability, cells were starved of glucose and treated with an agent that increases cAMP synthesis. In lower eukaryotes, the cAMP signal is often associated with nutrient deprivation, while in mammalian cells, cAMP accumulation is associated with hormonal signals that in turn can result in cell cycle arrest (11, 29, 49). The observed lability of Sp1 also required that the cells be deprived of glucosamine synthesis either as a result of glucose starvation or through the use of an inhibitor of GFAT, the rate-limiting enzyme in glucosamine synthesis. The combination of glucosamine deprivation and cAMP accumulation resulted in the formation of a markedly hypoglycosylated form of Sp1. Partial deglycosylation of Sp1 could also be reproduced in vitro upon mixing of a nuclear extract containing normally glycosylated Sp1 with an extract from cells treated with forskolin and a low level of glucose. This hypoglycosylated Sp1 could be observed only under conditions in which inhibitors of the proteasome were used to stabilize Sp1. These results therefore correlate the lability of Sp1 protein and its DNA-binding activity with its hypoglycosylation as a result of glucosamine deprivation and cAMP accumulation. These protease inhibitor results also suggest that the proteasome is involved in the degradation of this transcription factor.

The proteasome has already been established as vital in cell cycle control. The mitotic cyclins are labile proteins whose abundance is regulated during the cell cycle. The cyclins control a corresponding cyclin-dependent protein kinase and must be degraded to allow cell cycle progression. Generally, phosphorylation of the cyclins at specific residues is a signal that results in ubiquitination. The addition of this polyubiquitin chain targets the cyclins for degradation by the proteasome (1, 15, 37, 50). Lactacystin is a *Streptomyces*-derived antibiotic that specifically inactivates the proteasome protease(s) by covalent modification of a threonine residue thought to reside in the catalytic site of the protease (19). That Sp1 degradation could be blocked by this inhibitor is compelling evidence for a role for the proteasome in the control of Sp1 abundance.

The glycosylation state of Sp1 correlates with its stability against proteolytic degradation. However, the mechanisms by which cAMP causes hypoglycosylation and activates the degradation remain unclear. Each molecule of Sp1 has multiple glycosylation sites (25), and the number of sites modified represents the balance between *O*-GlcNAcase and *O*-GlcNAc transferase activities. cAMP accumulation could result in the activation of an *O*-GlcNAcase or the inactivation of an *O*-GlcNAc transferase, either directly or through modulation of UDP-GlcNAc synthesis. However, other mechanisms can be postulated. Sp1 is also phosphorylated (24), and some of the phosphorylations could occur at the same serine or threonine residues that are targeted for glycosylation. An example of this kind of switching is c-Myc; threonine-58 of c-Myc has been shown to be either O glycosylated or phosphorylated (9). The

accumulation of cAMP could result in the phosphorylation of Sp1 at sites that might then preclude its glycosylation. However, experiments to determine whether forskolin treatment would result in a net increase in Sp1 phosphorylation showed no change (data not shown). The finding that both Sp1 and the nuclear pore protein p62 became hypoglycosylated in response to glucose starvation and forskolin treatment suggests that a general change in the balance between *O*-GlcNAcase and *O*-GlcNAc transferase activities occurred. That the extract from cells treated with forskolin and a low level of glucose when mixed with an untreated extract could result in partial deglycosylation of Sp1 provides further evidence for a shift in this balance. Whether this shift towards deglycosylation results from enhancement of the *O*-GlcNAcase activity or inhibition of the *O*-GlcNAc transferase in the extract remains to be determined.

Our experiments have established a good correlation between the glycosylation state of Sp1 and its ability to be proteolytically degraded by a proteasome-like mechanism. That is, glucose and glucosamine block the ability of forskolin to induce both the hypoglycosylation and degradation of Sp1, whereas blockade of glucosamine synthesis is permissive for degradation. Hypoglycosylated Sp1 may then be more susceptible to degradation for several potential reasons, including a change in conformation or the exposure of recognition or modification sites that are otherwise occluded by *O*-GlcNAc. In addition, since other proteins (e.g., nuclear pore protein p62) appear to concurrently undergo deglycosylation with this treatment, we cannot rule out the possibility that it is the *O*-GlcNAc state of an as-yet-unidentified protein that results in the specific targeting of Sp1 for degradation. This latter possibility is compatible with the observation that the mixing of a hypoglycosylated nuclear extract with a normal extract results in the degradation of Sp1. Regardless of the mechanism, the connection between stability and the *O*-GlcNAc modification has precedence. The phosphorylation state and activity of elongation factor 2 in protein synthesis are controlled by an O-glycosylated 67-kDa protein. Under conditions of serum starvation, this protein is deglycosylated and then degraded (14). The loss of this protein results in the phosphorylation of elongation factor 2 and the inhibition of protein synthesis (6, 13). This broad control mechanism for general protein synthesis is similar to that postulated here for transcriptional control. We have shown that nutritional deprivation plus a cAMP signal result in the deglycosylation of Sp1. The loss of this protein by proteasome-like degradation would be expected to result in the down-regulation of transcription of the ubiquitously expressed genes whose expression depends on Sp1. The converse may also hold, in that this result might explain our prior observation that addition of either glucose or glucosamine to vascular smooth muscle cells results in the up-regulation of transcription from the Sp1-regulated transforming growth factor α promoter (39).

Each molecule of Sp1 contains an average of eight *O*-GlcNAc modifications (25). To date, the exact sites of these modifications have not been published, although our laboratory has located one of these sites in a transcriptional activation domain of the molecule (39a). From the location of this site, we presume that the modification may play some role in the control of transcription. We have also ascertained that some of the other modification sites are in domains of the molecule that subserve functions other than transcriptional activation. Since these sites of modification subserve different functions, it remains possible that the glycosylations at these sites are differentially regulated. Thus, crude measures of total Sp1 glycosylation may not necessarily correlate with specific functional changes. Thus, the finding that there is some correlation between the total level of Sp1 glycosylation and its stability against proteasome degradation is all the more remarkable.

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