

High glucose and insulin promote O-GlcNAc modification of proteins, including α -tubulin

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Walgren, Jennie L. E., Timothy S. Vincent, Kevin L. Schey, and Maria G. Buse. High glucose and insulin promote O-GlcNAc modification of proteins, including α -tubulin. *Am J Physiol Endocrinol Metab* 284: E424–E434, 2003. First published October 22, 2002; 10.1152/ajpendo.00382.2002.—Increased flux through the hexosamine biosynthesis pathway has been implicated in the development of glucose-induced insulin resistance and may promote the modification of certain proteins with O-linked N-acetylglucosamine (O-GlcNAc). L6 myotubes (a model of skeletal muscle) were incubated for 18 h in 5 or 25 mM glucose with or without 10 nM insulin. As assessed by immunoblotting with an O-GlcNAc-specific antibody, high glucose and/or insulin enhanced O-GlcNAcylation of numerous proteins, including the transcription factor Sp1, a known substrate for this modification. To identify novel proteins that may be O-GlcNAc modified in a glucose concentration/insulin-responsive manner, total cell membranes were separated by one- or two-dimensional gel electrophoresis. Selected O-GlcNAcylated proteins were identified by mass spectrometry (MS) analysis. MS sequencing of tryptic peptides identified member(s) of the heat shock protein 70 (HSP70) family and rat α -tubulin. Immunoprecipitation/immunoblot studies demonstrated several HSP70 isoforms and/or posttranslational modifications, some with selectively enhanced O-GlcNAcylation following exposure to high glucose plus insulin. In conclusion, in L6 myotubes, Sp1, membrane-associated HSP70, and α -tubulin are O-GlcNAcylated; the modification is markedly enhanced by sustained increased glucose flux.

insulin resistance; L6 myotubes; O-linked N-acetylglucosamine; heat shock protein 70

THE ADDITION OF A SINGLE N-acetylglucosamine (GlcNAc) moiety to serine or threonine residues in proteins (O-GlcNAcylation) is a common posttranslational modification. It is unlike other glycosylation events in that it occurs through an enzyme-catalyzed reaction in the cytosol and the nucleus rather than in the Golgi apparatus or the endoplasmic reticulum. O-GlcNAcylation is a dynamic and reversible process, regulated by the activities of O-GlcNAc transferase (37, 41) and N-acetylglucosaminidase (O-GlcNAcase) (18), which

have been characterized and cloned. Although there is not a well-defined consensus sequence for O-GlcNAcylation, the modified regions generally contain a proline residue and usually multiple serines and threonines. These sites are often identical to the motifs used by serine and threonine kinases. In fact, in some cases, O-GlcNAcylation is reciprocal with phosphorylation (4, 6), which may have important consequences on protein activity and signaling. In addition to its potential role in regulating phosphorylation, O-GlcNAcylation also appears to play a role in protein transcription and translation, nuclear targeting and transport, and protein degradation (reviewed in Refs. 5 and 25). More information about the function of O-GlcNAcylation is being discovered as the proteins that undergo this modification are being identified.

O-GlcNAcylation may play a role in certain disease states such as Parkinson's disease, Alzheimer's disease, and diabetes (5, 64). In patients with diabetes, sustained hyperglycemia leads to insulin resistance ("glucose toxicity") (57). This glucose-induced insulin resistance may reflect, at least in part, increased flux through the hexosamine biosynthesis pathway (HBSP), a quantitatively minor component of overall glucose disposal (42). HBSP is the obligatory source of essential building blocks for the glycosylation of proteins and lipids. The major product is UDP-GlcNAc, the obligatory substrate for the transfer of O-GlcNAc to proteins. Studies in rodents (27, 28) suggest a correlation between the development of insulin resistance and increased UDP-GlcNAc concentrations in muscle. The latter may enhance the O-GlcNAc modification of certain proteins (37, 25), which may contribute to insulin resistance. Insulin-sensitive tissues, such as skeletal muscle, respond to the hormone with acceleration of glucose transport, which reflects, in great part, increased expression of the insulin-responsive glucose transporter isoform GLUT4 at the cell membrane. GLUT4 translocation and vesicle fusion with the plasmalemma involve an intricate signaling pathway linking the insulin receptor tyrosine kinase to numerous

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adapter proteins, kinases, and vesicular and cytoskeletal proteins. Enhanced O-GlcNAc modification of participating proteins may affect the insulin response. For instance, impaired insulin signaling concomitant with increased O-GlcNAcylation of insulin receptor substrate (IRS)-1 and IRS-2 was reported in skeletal muscle of rats infused with glucosamine plus insulin (52), and enhanced O-GlcNAc modification of IRS-1 and β -catenin was observed in 3T3-L1 adipocytes rendered insulin resistant by treatment with an O-GlcNAcase inhibitor (63).

Coinfusion of insulin and glucosamine for 6 h increases UDP-GlcNAc in skeletal muscle and enhances the O-GlcNAc modification of numerous unidentified proteins (66). Transgenic mice overexpressing GLUT1 in skeletal muscle exhibit chronically increased glucose flux and increased UDP-GlcNAc concentrations in muscle (3). Although basal glucose transport is markedly increased, it is resistant to further stimulation by insulin, although GLUT4 expression is normal (20). Muscles of these transgenic mice show two- to three-fold enhancement of the O-GlcNAc modification of numerous membrane-associated proteins, including proteins that appear to be associated with GLUT4 (3). In the present study, we used L6 myotubes as a model system for skeletal muscle, aiming to identify proteins with enhanced O-GlcNAc modification in response to sustained increased glucose flux.

MATERIALS AND METHODS

Materials. Unless otherwise noted, materials were purchased from Sigma Chemical (St. Louis, MO) and were of the highest quality available. α -Minimal essential growth medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO Invitrogen (Grand Island, NY). Human recombinant insulin was a gift from Lilly Research Laboratories (Indianapolis, IN). Rabbit polyclonal anti-Sp1 (PEP-2), anti- α -tubulin antibodies, and monoclonal anti-HSP70 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit polyclonal anti-HSP70 was obtained from Upstate Biotechnologies (Lake Placid, NY). Monoclonal RL-2 anti-O-GlcNAc antibody was purchased from ABR (Golden, CO). Anti-O-GlcNAc (110.6) antibody was a kind gift of Dr. G. W. Hart (7). Rabbit polyclonal anti-Sp1 (3517) was a kind gift of Dr. J. E. Kudlow (23). Nitrocellulose membranes were obtained from Osmonics (Westborough, MA). Ampholytes, immobilized pH gradient (IPG) isoelectric focusing strips, IPG strip Criterion gels, and Sypro orange stain were purchased from Bio-Rad (Hercules, CA). E-zinc stain was obtained from Pierce (Rockford, IL). Sequencing-grade acetonitrile, formic acid, and trifluoroacetic acid (TFA) were purchased from Fisher Chemical (Pittsburgh, PA). Sequencing-grade trypsin was obtained from Promega (Madison, WI), and C-18 ZipTips were from Millipore (Bedford, MA).

L6 cell culture. Cryopreserved L6 myocytes/myoblasts were a kind gift of Dr. Amira Klip. The cells were passaged according to Mitumoto and Klip (44) in α -MEM growth medium containing 100 U/ml penicillin, 100 μ g/ml streptomycin, 250 μ g/ml amphotericin, and 10% fetal calf serum. To obtain differentiated myotube cultures, cells were seeded at 2×10^4 cells/ml into α -MEM containing 2% fetal calf serum and maintained in culture for ≥ 1 wk postconfluence before experimentation.

L6 cell treatment and fractionation. Once differentiated, cells were incubated for 18 h in DMEM containing 1% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 250 μ g/ml amphotericin, 4 mM glutamine, 1 mM sodium pyruvate, and either 5 or 25 mM glucose in the presence or absence of 10 nM insulin. Glucosamine-treated cells were incubated in medium containing 5 mM glucose and 2.5 mM glucosamine in the presence or absence of 10 nM insulin.

In Sp1 studies, total cell extracts were prepared as follows. L6 cells were rinsed with PBS, scraped into 1 ml of lysis buffer containing 10 mM Tris (pH 7.5), 600 mM NaCl, 1 mM dithiothreitol (DTT), 50 mM GlcNAc, 330 μ M calpain inhibitor 1 (N-acetyl-leu-leu-norleu-al, LLnL), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μ g/ml each of leupeptin, aprotinin, and pepstatin A, frozen and thawed three times, and centrifuged at 1,000 g for 1 min. The supernatant was homogenized with a Dounce homogenizer, mixed for 30 min at 4°C, and centrifuged at 10,000 g for 10 min. The supernatant was analyzed for protein content and used for SDS-PAGE or immunoprecipitation.

For studies examining O-GlcNAcylation of membrane-associated proteins, plates were rinsed with cold PBS, and cells were scraped into 200 μ l of preparation buffer containing 10 mM Tris (pH 7.4), 250 mM sucrose, 1 mM EDTA, 330 μ M LLnL, 1 mM PMSF, and 10 μ g/ml leupeptin and aprotinin. Cells were homogenized with a Dounce homogenizer, and cell debris was pelleted at 14,000 rpm in a microcentrifuge. The supernatant was centrifuged at 174,000 g for 2 h at 4°C. The resulting supernatant was designated the cytosol. The pellet was resuspended in 125 μ l of reconstitution buffer containing 50 mM HEPES (pH 7.3), 150 mM NaCl, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 330 μ M LLnL, and 10 μ g/ml leupeptin and aprotinin, and was designated the membrane fraction. Membrane fractions were mixed at 4°C for ≥ 30 min to allow for maximal solubilization and then centrifuged at 14,000 rpm for 5 min to remove insoluble material. The supernatants were analyzed for protein content and used for SDS-PAGE or two-dimensional gel electrophoresis (2-DE) or for immunoprecipitation.

Immunoprecipitations. Immunoprecipitation of Sp1 was carried out overnight in 1 ml of lysis buffer diluted to normal salt (150 mM NaCl) concentration containing 500–600 μ g of total cell extract protein, 25 μ l of packed protein A-Sepharose beads, and 2 μ g/ml rabbit polyclonal anti-Sp1 antibody (Santa Cruz Biotechnologies). Immunoprecipitation of other proteins of interest from membrane fractions was carried out overnight in 0.5–1 ml of membrane reconstitution buffer diluted to 0.5% CHAPS containing 300–500 μ g of membrane protein, 25–40 μ l of packed protein A-Sepharose (Sigma) or anti-IgM-agarose (Sigma) beads, and 1–2 μ g of primary antibody. Rabbit polyclonal antibodies were used for the immunoprecipitation of HSP70 (Upstate Biotechnologies) and α -tubulin (Santa Cruz Biotechnologies). An anti-O-GlcNAc (IgM) antibody (110.6) (7) was used to immunoprecipitate O-GlcNAcyated proteins.

The following morning, the beads were centrifuged at 2,000 rpm for 2–3 min, and the supernatant was stored at -80°C in Laemmli's buffer to verify the efficiency of immunoprecipitation by immunoblot analysis. Beads were washed three times with either wash buffer containing 10 mM Tris (pH 7.5), 50 mM GlcNAc, and 0.1% Triton X-100 (for total cell extracts) or with membrane reconstitution buffer containing 0.5% CHAPS (for membrane preparations). In Sp1 O-GlcNAcylation studies, the beads were resuspended in 60 μ l of 10 mM Tris (pH 8.3), 10 mM DTT, and 10% SDS and were mixed for 20 min at room temperature. Samples were then incubated with 25 mM iodoacetic acid for 30 min with agita-

tion. After acid elution, Laemmli's buffer was added to each sample, and, after thorough mixing, beads were spun down, and the supernatant was collected for SDS-PAGE analysis. For membrane immunoprecipitates used in 2-DE, the beads were resuspended in 50 μ l of 2-DE CHAPS buffer containing 8 M urea, 4% CHAPS, 0.3% DTT, 0.5% pH 3–10 ampholytes, and a trace of bromphenol blue dye. The samples were mixed for ≥ 1.5 h at room temperature and centrifuged, and the supernatant was loaded onto isoelectric focusing strips.

SDS-PAGE and 2-DE. For Sp1 experiments, proteins were separated by SDS-PAGE (7% polyacrylamide) and were transferred to nitrocellulose. Blots were developed using the mouse monoclonal RL-2 anti-O-GlcNAc antibody (ABR) or a rabbit Sp1 antibody (23).

For 2-DE analysis, samples were cup loaded onto Bio-Rad IPG isoelectric focusing strips (various pH gradients depending on protein of interest) at 300 V for 3 h. Voltage was gradually increased to 3,500 V over the next hour, and strips were focused for an additional 18 h at 3,500 V. After being focused, strips were incubated for 15 min in 2-DE equilibration buffer containing 50 mM Tris·HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS, a trace of bromphenol blue dye, and 15 mg/ml DTT. This buffer was removed, and the strips were incubated in the same buffer containing 18 mg/ml iodoacetamide. After this equilibration, the proteins in the strips were separated on either 10–20% gradient or 10% Bio-Rad Criterion IPG strip Tris gels (preequilibrated with SDS). Gels were then either stained immediately with E-zinc stain or Sypro orange or transferred to nitrocellulose for Western blot analysis. Bands of interest were excised from protein-stained gels, destained, and either digested immediately with trypsin or frozen at -80°C for later analysis.

Mass spectrometry: in-gel trypsin digestion. Excised gel bands were incubated in 100 mM ammonium bicarbonate for 1 h. This was removed, and 50 μ l of a solution containing 50% 100 mM ammonium bicarbonate and 50% acetonitrile were placed on each sample and then removed. Gel pieces were then cut into 1×1 -mm fragments and placed into tubes with 100 μ l of acetonitrile. After 15 min of incubation, acetonitrile was removed, and samples were dried in a Speed-Vac. Ten microliters of 10 ng/ml sequencing-grade trypsin were placed on each of the samples and allowed to soak into the gel pieces for 10 min. Enough 100 mM ammonium bicarbonate solution to cover the gel pieces was placed into each tube, and the samples were incubated overnight at 37°C . The following morning, the solution from each tube was removed, saved, and replaced with a similar volume of 50% acetonitrile and 5% formic acid, and the samples were sonicated for 20 min. The solution was removed from the gel pieces and saved, and the procedure was repeated. The three extracts were combined and dried for 2–3 h in a Speed-Vac.

Matrix-assisted laser desorption ionization (MALDI) analysis was performed on a Voyager-DE Biospectrometry Workstation (Applied Biosystems). Samples were resuspended in 10% acetonitrile and 0.1% TFA and desalted using a C-18 Zip-Tip. After a wash with 0.1% TFA, peptides were eluted in 2 μ l of 50% acetonitrile and 0.1% TFA for MALDI analysis and 50% acetonitrile and 2% acetic acid for nanospray analysis. Samples were mixed 1:3 (vol/vol) with the matrix α -cyano-4-hydroxycinnamic acid (50 mM) in 70% acetonitrile and 0.1% TFA. The peptide/matrix solutions were placed on a gold plate and allowed to dry. A minimum of 200 laser shots were averaged to produce the mass spectra. The instrument was calibrated with peptide standards from Applied Biosystems. Laser power was optimized for signal intensity and resolution and varied between 1,700–1,950. Protein identification based on MALDI signals, known as peptide mass

fingerprinting, was accomplished using the Internet software ProFound (http://129.85.19.192/profound_bin/WebProFound.exe).

Mass spectrometry (MS)/MS spectra were obtained using a custom nanospray source on an LCQ Classic ion trap mass spectrometer (Finnigan) or a Protana nanospray source on a Q-STAR quadrupole/time-of-flight mass spectrometer (Applied Biosystems). A tryptic digest sample (1–2 μ l) in 50% acetonitrile–2% acetic acid was loaded into a pulled-glass capillary, and $\sim 1,200$ V were applied to the capillary tip to initiate a spray. Peptides of interest were isolated according to their mass-to-charge ratio and fragmented to produce a tandem (MS/MS) mass spectrum. Typically, 20–50 scans were averaged to produce one MS/MS spectrum. Protein identification based on short stretches of sequence interpreted from MS/MS spectra was accomplished using the Internet software PepSea (http://195.41.108.38/PA_PeptidePatternForm.html).

Statistical analyses. Means \pm SE are shown. The significance of differences between means was evaluated by two-tailed, unpaired Student's *t*-test and by one-way analysis of variance (ANOVA; Microsoft Excel 2000; Redmond, WA). *P* < 0.05 was considered significant.

RESULTS

O-GlcNAcylation of Sp1. The ability of L6 myotubes to O-GlcNAcylate the transcription factor Sp1 was examined using immunoprecipitation/immunoblotting. Sp1 was immunoprecipitated from total cellular proteins using the anti-Sp1 rabbit polyclonal antibody (PEP-2). O-GlcNAcylation was determined by immunoblotting with RL-2 antibody. We found that Sp1 is O-GlcNAcylated in L6 myotubes. When incubated in 5 mM glucose, the addition of 10 nM insulin enhanced Sp1 O-GlcNAcylation approximately threefold (Fig. 1, A and C). Incubation in 25 mM glucose (without insulin) further enhanced Sp1 O-GlcNAcylation (~ 4 -fold increase compared with 5 mM glucose without insulin). Concurrent treatment with insulin and 25 mM glucose did not result in additional enhancement of the modification. The observed changes in O-GlcNAcylation were not due to differences in Sp1 protein levels (Fig. 1, B and D).

Identification of O-GlcNAcylated proteins in L6 membrane fractions. In view of previous studies that indicated that chronically increased glucose flux promoted the O-GlcNAc modification of membrane proteins in skeletal muscle (2), we next examined the O-GlcNAcylation of membrane-associated proteins resolved on 7% SDS-PAGE and immunoblotted with RL-2 (Fig. 2A). Numerous proteins appeared to be O-GlcNAcylated in L6 membrane fractions. Incubation in media containing 25 mM glucose or 2.5 mM glucosamine added to 5 mM glucose enhanced the O-GlcNAc modification of several protein bands (compared with 5 mM glucose) in the absence of insulin. Insulin markedly enhanced O-GlcNAcylation in cells incubated in 5 mM glucose and, unlike Sp1, further increased the signal from several proteins in cells treated with either 25 mM glucose or 2.5 mM glucosamine.

In an effort to identify some of these O-GlcNAcylated proteins, we employed 2-DE to obtain greater resolution of the membrane proteins. Samples from unstimu-

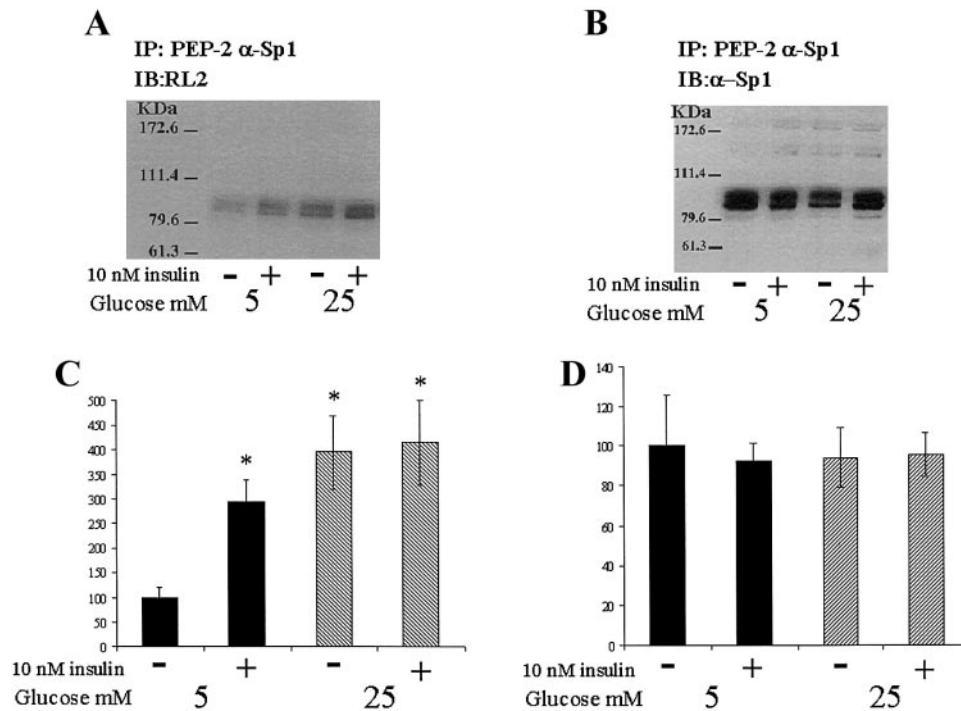
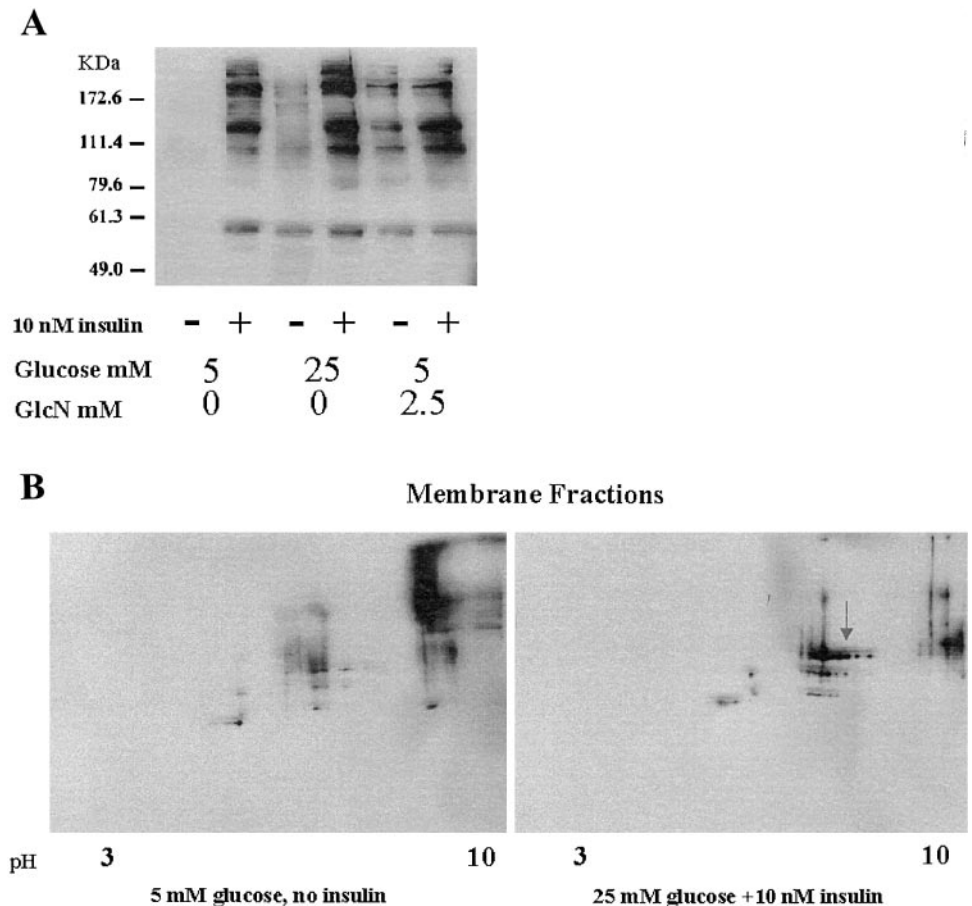


Fig. 1. Sp1 is O-linked glycosylated on Ser/Thr with single N-acetylglucosamine (O-GlcNAcylated) in L6 myotubes, and this modification is enhanced by exposure of the cells to insulin or high glucose. L6 myotubes were treated for 18 h with either 5 mM glucose or 25 mM glucose in the presence or absence of 10 nM insulin. Total cellular proteins were isolated, and Sp1 (α -Sp1) was immunoprecipitated (IP) overnight using a rabbit polyclonal antibody (PEP-2). Proteins were eluted with iodoacetic acid, separated by 7% SDS-PAGE, and transferred to nitrocellulose. Membranes were immunoblotted (IB) with a monoclonal anti-O-linked N-acetylglucosamine (O-GlcNAc) antibody (RL-2; A), stripped, and then immunoblotted with a second anti-Sp1 antibody (B). Data shown are representative of 3–4 independent experiments. Densitometric analyses of all experiments are represented in C (RL-2 immunoblot) and D (α -Sp1 immunoblot). * $P < 0.05$, significantly different from signal in 5 mM glucose – insulin, by Student's *t*-test or ANOVA.

Fig. 2. O-GlcNAcylation of L6 membrane-associated proteins is enhanced following exposure of the cells to insulin, high glucose, or glucosamine. A: L6 myotubes were treated for 18 h with either 5 mM glucose, 25 mM glucose, or 2.5 mM glucosamine + 5 mM glucose each \pm 10 nM insulin. Total membrane-associated proteins were separated by 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the O-GlcNAc-specific antibody RL-2. Data are representative of >10 independent experiments. B: L6 myotubes were treated for 18 h with either 5 mM glucose or 25 mM glucose + 10 nM insulin. Total membrane-associated proteins were separated by 2-dimensional (2-D) electrophoresis [pH 3–10 isoelectric focusing (IEF) gradient and 10% SDS-PAGE], transferred to nitrocellulose, and immunoblotted with the O-GlcNAc-specific antibody RL-2. Data are representative of 3 independent experiments.



lated (5 mM glucose) and stimulated (25 mM glucose + insulin) cells were resolved in duplicate on pH 3–10 gradient 10% SDS-PAGE two-dimensional gels for protein staining and immunoblotting. Immunoblotting with RL-2 (Fig. 2B) revealed enhanced O-GlcNAcylation in proteins from stimulated cells. A trailing pattern often observed with glycosylated proteins was also noted. Protein spots corresponding to immunostained proteins were cut from the duplicate protein-stained gel for mass spectrometric analysis.

HSP70 is O-GlcNAcylated in L6 myotubes. Protein samples that corresponded to immunostained proteins from L6 membrane fractions were excised from two-dimensional gels and digested with trypsin. Proteins were extracted and analyzed by MALDI. A representative spectrum is shown in Fig. 3A. The ProFound software program (PROWL, Rockefeller University) was used to analyze the tryptic fragments. Mining of the database revealed a match with the HSP70 family of proteins.

To validate the identity of this membrane-associated protein, we sequenced tryptic digests with the same fragment pattern by use of a custom nanospray source on an LCQ Classic (Finnigan). We sequenced fragments of HSP70, including residues 115–118 (YPEE) from the 1,630.9 molecular weight (MW) tryptic fragment, residues 180–187 (AIAYGLDR) from the 1,687.9-MW tryptic fragment, and residues 39–43 (PSYVA) from the 1,487.6-MW tryptic fragment, the MS/MS spectrum of which is shown in Fig. 3B.

α -Tubulin is O-GlcNAcylated in L6 myotubes. Protein samples that corresponded to immunostained proteins from L6 membrane fractions were excised from 10% SDS-PAGE gels and digested with trypsin. Proteins were extracted and analyzed by MALDI. A representative spectrum is shown in Fig. 4A. The ProFound software program was used to analyze the tryptic fragments. Mining of the database revealed a match with α -tubulin.

To validate the identity of this membrane-associated protein, we sequenced tryptic digests with the same fragment pattern by use of a Protana nanospray source on a Q-STAR instrument (Applied Biosystems). We sequenced fragments of α -tubulin, including residues 274–277 (PVIS) from the 1,758.1-MW tryptic fragment, residues 73–79 (TVIDEVR) from the 1,701.9-MW tryptic fragment, and residues 253–261 (TNLVPYP) from the 2,410.7-MW tryptic fragment, the MS/MS spectrum of which is shown in Fig. 4B.

O-GlcNAcylation of HSP70 and α -tubulin is enhanced in high glucose-treated L6 myotubes. The ability of L6 cells to O-GlcNAcylate HSP70 and α -tubulin was further confirmed using immunoprecipitation/immunoblotting. Total membranes were isolated from L6 cells treated with either normal glucose (5 mM) alone or high glucose (25 mM) plus 10 nM insulin. Proteins were separated on 7% SDS-PAGE gels and transferred to nitrocellulose. Resulting immunoblots using either the RL-2 anti-O-GlcNAc antibody or monoclonal anti-HSP70 revealed that O-GlcNAcylation of proteins in

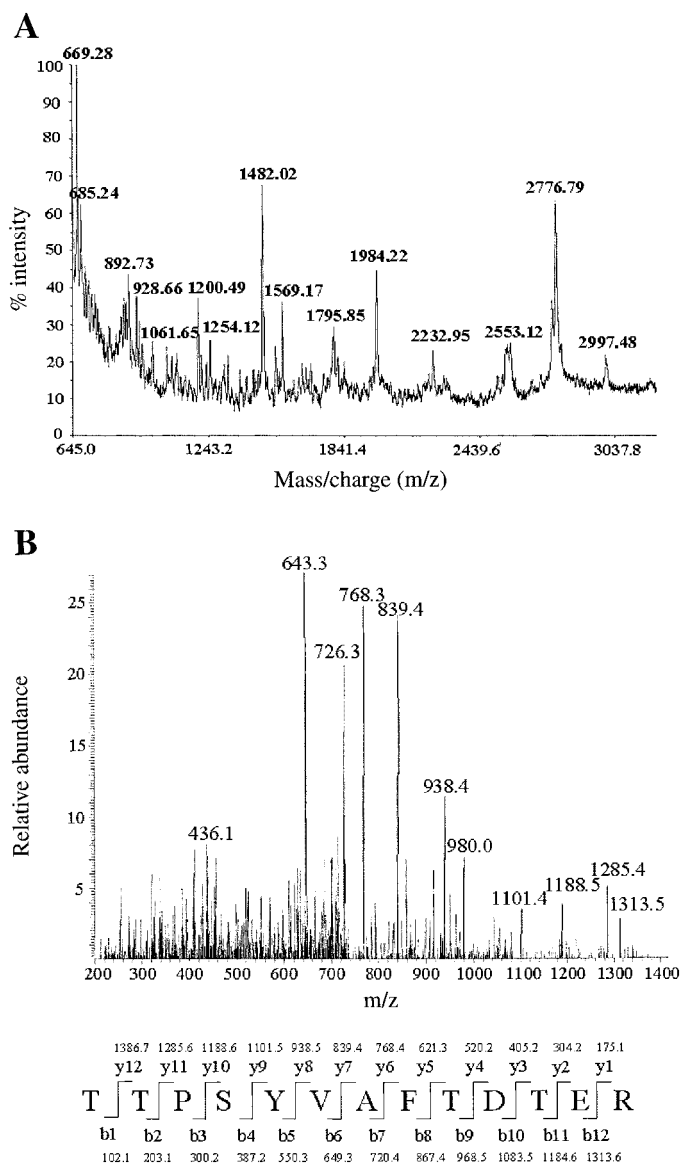


Fig. 3. A member of the heat shock protein (HSP)70 family of proteins is O-GlcNAcylated in L6 myotubes. Protein spots on zinc-stained 2-D gels that corresponded to immunostained proteins on concurrently analyzed 2-D RL-2 immunoblots from identical samples were excised. These excised proteins were digested with trypsin, extracted from the gel, and analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. **A**: tryptic fragments detected in the MALDI spectrum were analyzed using the ProFound program (PROWL Web-based software) and revealed that this protein was likely a member of the HSP70 family. The spectrum shown is representative of membranes from cells incubated in 5 mM glucose; 25 mM glucose and glucosamine samples revealed similar patterns. Similar digests were sequenced using a custom nanospray source on an LCQ Classic. **B**: representative mass spectrometry (MS)/MS spectrum is shown. This spectrum corresponds to residues 39–43 (PSYVA) from the 1,487.6-MW tryptic peptide fragment in rat HSP70.1/2 (sptrembl no. Q07439; NCBI no. 729767).

the molecular weight range of HSP70 increased following exposure of the cells to high glucose, whereas protein levels of HSP70 did not change significantly (Fig. 5A). We also immunoprecipitated HSP70 with a polyclonal antibody, and separated these immunoprecipitates on pH

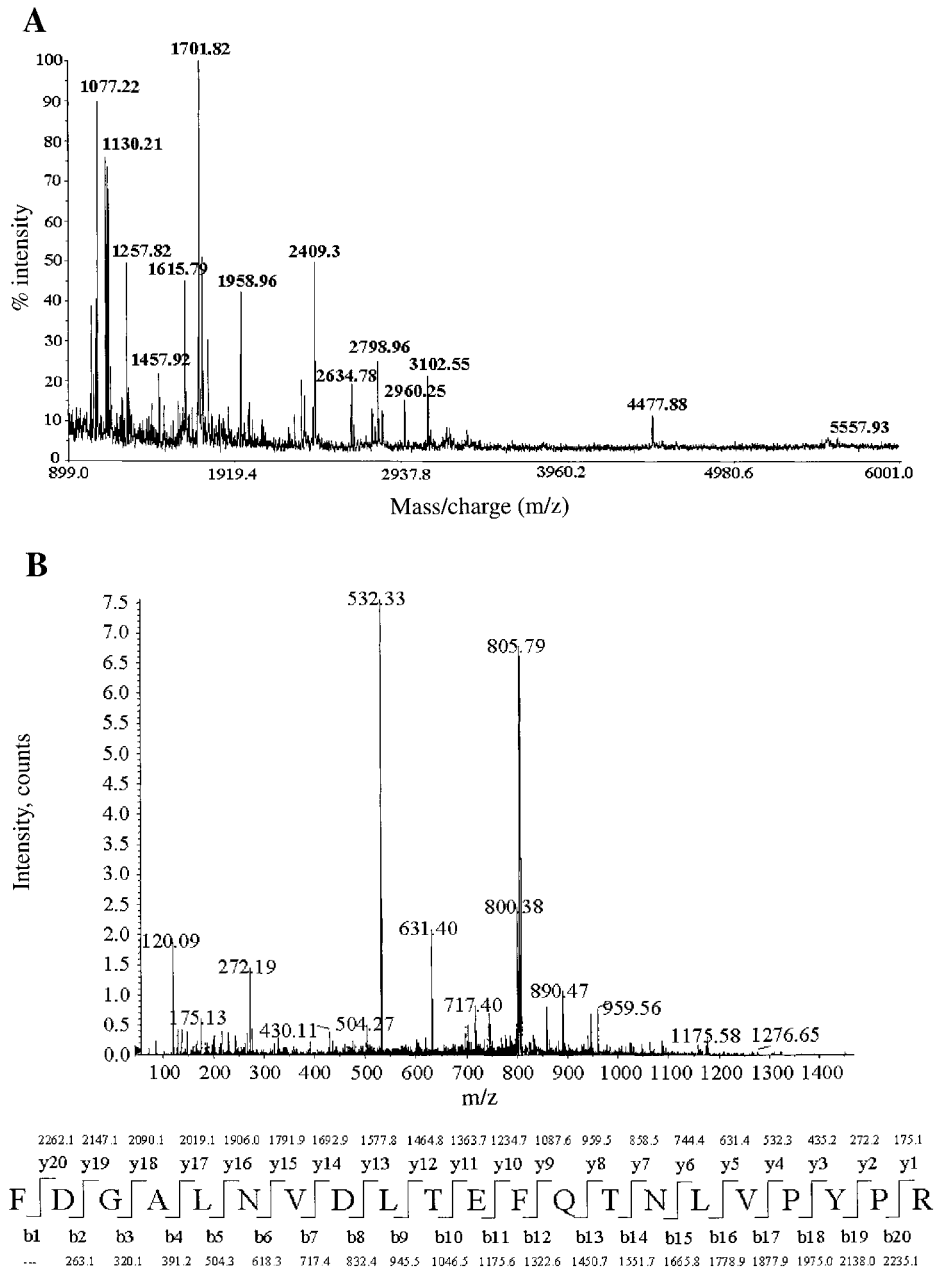


Fig. 4. α -Tubulin is O-GlcNAcylated in L6 myotubes. Protein spots on zinc-stained SDS-PAGE gels that corresponded to immunostained proteins on concurrently analyzed RL-2 immunoblots from identical samples were excised, digested with trypsin, extracted from the gel, and analyzed by MALDI MS. A: tryptic fragments detected in the MALDI spectrum were analyzed using the ProFound program, which revealed that this protein was likely α -tubulin. The spectrum shown is representative of membranes from cells incubated in 25 mM glucose + insulin; 5 mM glucose samples revealed a similar pattern. Similar digests were sequenced using a Protana nanospray source on a Q-STAR instrument. B: representative MS/MS spectrum is shown. This spectrum corresponds to residues 253–261 (TNLVPYP) from the 2,410.7-MW tryptic fragment in rat α -tubulin (NCBI no. 11560133).

5–8 two-dimensional gels. After transferring these immunoprecipitated proteins to nitrocellulose, we immunoblotted again with either monoclonal anti-HSP70 or RL-2. The immunoblots resolved several proteins that reacted with the anti-HSP70 antibody. There was very little change in protein levels between the 5 and 25 mM glucose treatment groups, except for a small decrease in immunostaining in the proteins with lower isoelectric point (pI). However, when these blots were probed with RL-2, there was a substantial increase in immunostaining of several protein spots with a greater pI in the high-glucose treatment group (Fig. 5B).

To examine the O-GlcNAcylation of α -tubulin and compare protein levels of α -tubulin between treatment groups, total cellular extracts were made from cells treated with either normal (5 mM) glucose or high (25

mM) glucose plus 10 nM insulin. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose. Immunoblots using either the RL-2 anti-O-GlcNAc antibody or monoclonal anti- α -tubulin revealed that O-GlcNAcylation of proteins in the molecular mass range of α -tubulin increased following exposure of the cells to high glucose plus insulin, whereas protein levels of α -tubulin did not change significantly (Fig. 6).

To further confirm that HSP70 and α -tubulin were O-GlcNAcylated and that this modification was increased after high glucose exposure, O-GlcNAcylated proteins were immunoprecipitated from membrane fractions using the anti-O-GlcNAc (110.6) mouse IgM antibody, and the resulting proteins were resolved on two-dimensional gels (pH 3–10 gradient; 10–20% gra-

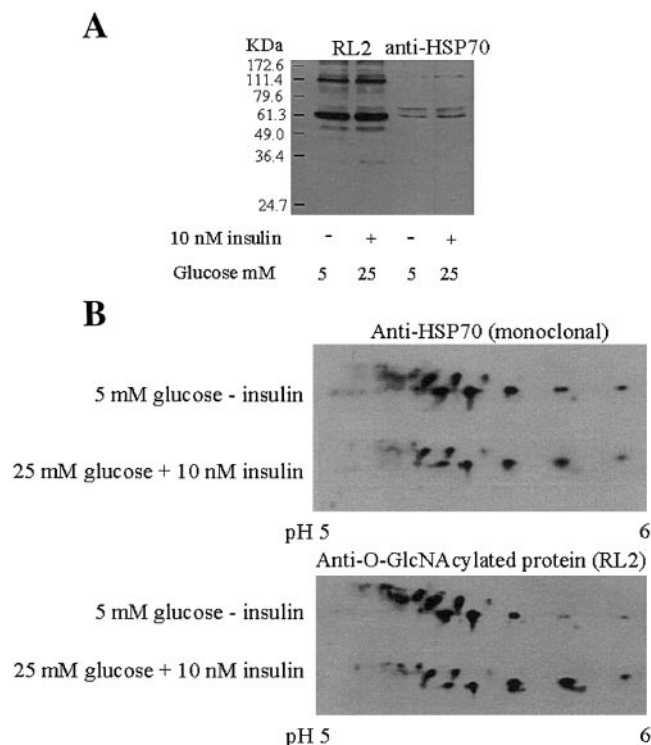


Fig. 5. O-GlcNAcylation of HSP70 in L6 total membrane fractions. L6 myotubes were treated for 18 h with either 5 mM glucose or 25 mM glucose + 10 nM insulin. Membrane fractions were isolated and either subjected directly to 10% SDS-PAGE and transferred to nitrocellulose (A) or immunoprecipitated with polyclonal anti-HSP70 antibody. Immunoprecipitated proteins were separated by 2-D gel electrophoresis (pH 5–8 IEF gradient and 10–20% gradient SDS-PAGE) and transferred to nitrocellulose (B). Membranes were immunoblotted with either monoclonal anti-HSP70 or RL-2 (anti-O-GlcNAc) antibodies. Data are representative of 5–7 independent experiments.

dient SDS-PAGE). The resulting blot was probed with either rabbit polyclonal anti-HSP70 or anti- α -tubulin. Both HSP70 and α -tubulin demonstrated an increase in O-GlcNAcylation after cells were treated with high glucose (25 mM) and 10 nM insulin (Fig. 7). The increase in anti-O-GlcNAc antibody-precipitable α -tubulin in response to treatment was remarkable, on the order of >10-fold.

DISCUSSION

Increased UDP-GlcNAc in skeletal muscle is associated with insulin resistance in numerous experimental models (3, 27, 28). It may promote the O-GlcNAc modification of proteins in part by mass action and in part as an allosteric regulator of O-GlcNAc transferase, enhancing the affinity of the enzyme for certain protein substrates (38). Indeed, enhanced O-GlcNAc modification of numerous proteins has been demonstrated in skeletal muscle in vivo following sustained infusions of glucosamine and insulin (52, 66), a condition that causes insulin resistance and large increases in UDP-GlcNAc. In transgenic mice overexpressing GLUT1 in muscle, chronically increased glucose flux into muscle is associated with insulin resistance of muscle glucose

transport, approximately twofold increases in UDP-GlcNAc, and markedly increased O-GlcNAc modification of numerous membrane-associated muscle proteins. These proteins coimmunoprecipitated with GLUT4 (the insulin-responsive glucose transporter), suggesting that they may be involved in the translocation to or fusion with the cell membrane of GLUT4-containing vesicles (2). A major objective of the present work was to identify some of the membrane-associated proteins that responded to increased glucose flux with enhanced O-GlcNAc modification with the use of L6 myotubes as a model for skeletal muscle.

Preincubation in 25 mM glucose plus 100 nM insulin for 24 h impairs subsequent acute insulin-stimulated GLUT4 translocation in L6 myotubes. The proximal insulin-signaling cascade is downregulated in terms of both protein expression of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) and phosphorylation (activation) of IR, IRS-1, and phosphatidylinositol 3-kinase (PI 3 kinase) and Akt. Concomitantly, the intrinsic activity of GLUT4 appears to be upregulated, which in part compensates for the translocation defect and may reflect enhanced p38 mitogen-activated protein kinase activity (29). Insulin-stimulated GLUT4 translocation is also impaired in muscles of insulin-resistant transgenic mice, which overexpress glutamine fructose-6-phosphate amidotransferase (the rate-limiting enzyme of HBSP) in skeletal muscle and adipocytes (9). On the other hand, in GLUT1-overexpressing muscles, insulin-stimulated GLUT4 translocation to the plasma membrane appears to be unimpaired (as assessed by exofacial photolabeling and by electron microscopy) (24), and the impaired insulin-

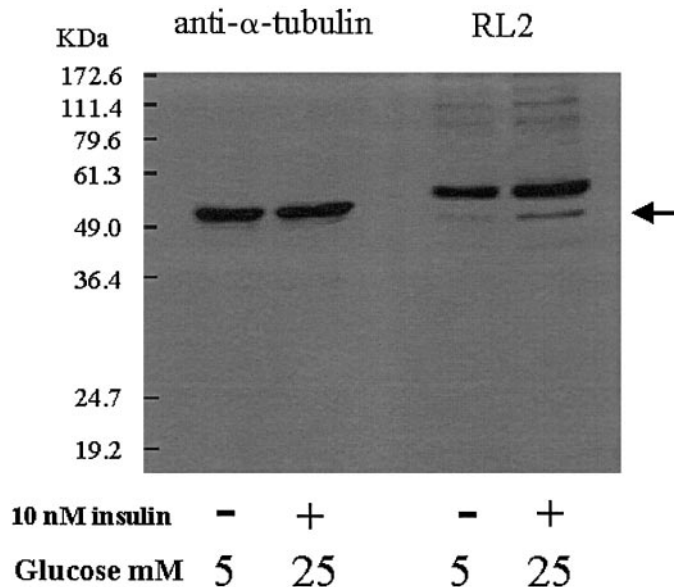


Fig. 6. O-GlcNAcylation of α -tubulin in L6 total cellular extracts. L6 myotubes were treated for 18 h with either 5 mM glucose or 25 mM glucose + 10 nM insulin. Total cellular extracts were subjected to 10% SDS-PAGE and transferred to nitrocellulose. Membranes were immunoblotted with either monoclonal anti- α -tubulin or RL-2 (anti-O-GlcNAc) antibodies. Data are representative of 3 independent experiments.

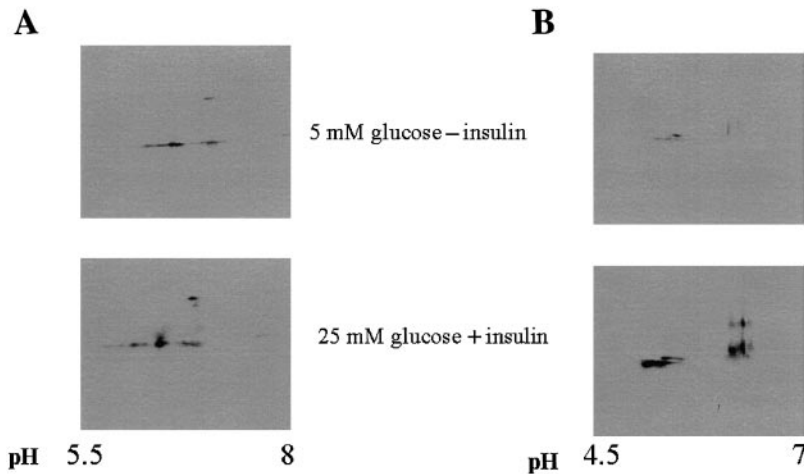


Fig. 7. *O*-GlcNAcylation of HSP70 and α -tubulin is increased in L6 myotubes treated with high glucose and insulin. L6 myotubes were treated for 18 h with either 5 mM glucose – insulin or 25 mM glucose + 10 nM insulin. Membranes were isolated, and *O*-GlcNAcylated proteins were immunoprecipitated with IgM anti-*O*-GlcNAc antibody 110.6. Immunoprecipitated proteins were separated by 2-D gel electrophoresis (pH 3–10 IEF gradient and 10–20% gradient SDS-PAGE) and transferred to nitrocellulose. Membranes were immunoblotted with either rabbit polyclonal anti-HSP70 (A) or rabbit polyclonal anti- α -tubulin (B). Data are representative of 3 independent experiments.

stimulated glucose transport is consistent with inappropriate insertion of GLUT4 into the plasma membrane and/or decreased GLUT4 intrinsic activity. The *O*-GlcNAc modification of numerous proteins is increased both in L6 cells incubated in high glucose plus insulin and in GLUT1-overexpressing muscles. There are many differences between the two models; the difference in the chronic ambient insulin concentration, which is <0.1 nM in serum of GLUT1-transgenic mice, deserves comment. In 3T3-L1 adipocytes, sustained incubation with insulin 100 nM or higher down-regulates the proximal insulin-signaling cascade (53, 54), whereas this pathway is not affected by sustained exposure to low or high glucose in the presence of 0.6 nM insulin (47, 48). The insulin resistance of glucose transport that develops in the presence of 25 mM glucose and 0.6 nM insulin in 3T3-L1 adipocytes appears to be consistent with modest impairment of GLUT4 translocation (49) and, in addition, possible dysregulation of GLUT4 insertion into the cell membrane and/or decreased GLUT4 intrinsic activity (47, 49). Insulin-mediated Akt activation is impaired distally to PI 3-kinase activation in these insulin-resistant 3T3-L1 adipocytes (48) as well as in rat skeletal muscle after incubation in high glucose (39). Which of these mechanisms, including protein modifications such as *O*-GlcNAcylation, is primarily responsible for glucose-induced insulin resistance of glucose transport in skeletal muscle, in vivo, in rodents or humans (57) is yet to be determined.

Sp1 is a transcription factor, which, along with several other RNA polymerase II transcription factors, is known to be *O*-GlcNAc modified at multiple sites (12). To characterize the regulation of the *O*-GlcNAc modification in L6 myotubes, in initial experiments we examined whether these cells responded to insulin and/or exposure to high glucose by increasing the *O*-GlcNAc modification of Sp1. In addition to regulating the expression of housekeeping genes, hormones, and metabolic enzymes such as acetyl-CoA carboxylase (10), leptin (8, 15), fatty acid synthase, and ATP citrate-lyase (15, 16), enhanced transcriptional activity of Sp1 has been associated with glucose-induced second-

ary diabetic complications through enhancement of transforming growth factor (TGF)- α (58), TGF- β , and plasminogen activator inhibitor (PAI)-1 transcription (12, 33, 36). Functional effects of *O*-GlcNAcylation on Sp1 include protection from protein degradation (22) and disruption of the interaction of Sp1 with TATA binding-associated factor 110 and holoSp1 (55).

In the present study, *O*-GlcNAcylation of Sp1 in L6 cells was apparent and was enhanced when glucose uptake was increased either through addition of insulin in normal (5 mM) glucose medium or after exposure of the cells to high glucose (25 mM). Total expression of Sp1 protein was unchanged under all conditions (Fig. 1B). In a previous report (22), *O*-GlcNAcylation enhanced Sp1 protein expression by slowing Sp1 degradation. However, the experimental conditions in Ref. 22 were markedly different from ours. Normal rat kidney-derived cells were incubated for 39 h in glucose-free medium, and forskolin was added during the last 24 h to elevate intracellular cAMP. The latter promoted Sp1 deglycosylation and Sp1 degradation only in glucose-starved cells; it was ineffective in cells incubated in 5 mM glucose or in 5 mM glucosamine. In our experiments, control L6 cells were incubated in 5 mM glucose. Possibly, the site that stabilizes Sp1 protein is already *O*-GlcNAc modified under physiological conditions, and the enhanced *O*-GlcNAcylation in response to increased glucose flux may involve additional Sp1 sites. Similarly, in other studies where incubation in high glucose (compared to physiological glucose) promoted Sp1 *O*-GlcNAcylation and the induction of TGF- β 1 and PAI-1, no effect on Sp1 protein expression was observed (12). Hyperglycemia-mediated activation of the PAI-1 promoter was blocked by mutating its two Sp1-binding sites or by decreasing HBSP activity, which reduced UDP-GlcNAc availability (12). DNase I protection studies failed to demonstrate increased DNA binding of *O*-GlcNAcylated Sp1, although its transcriptional efficiency appeared to be markedly enhanced (32). *O*-GlcNAcylation of Sp1 decreases its *O*-phosphorylation (12, 21). Because *O*-GlcNAc modification of Ser⁴⁸⁴ in the glutamine-rich transactivation domain of Sp1 inhibits known Sp1 protein interactions

(55), it has been proposed that enhanced O-GlcNAcylation may increase Sp1-mediated transactivation by inhibiting the binding of nuclear repressor proteins, such as p74 and the retinoblastoma-related protein p107 (11, 46), to the amino terminus of the Sp1 transactivation domain (12). However, O-GlcNAcylation of the Sp1 activation domain peptide or of native Sp1 decreases its transcriptional efficiency in an in vitro assay system (65). Thus, although there is apparent consensus that O-GlcNAcylation modulates Sp1 transcriptional activity, the mechanisms of regulation are controversial. As with Sp1, enhanced O-GlcNAc modification of HSP70 or of α -tubulin had no significant effect on the concentration of these proteins in our study (Figs. 5 and 6).

We identified HSP70 as one of the membrane-associated, high-glucose/insulin-responsive O-GlcNAcylated proteins in L6 myotubes. The HSP70 family is one of several ubiquitous molecular chaperone protein families. HSP70s are generally cytosolic but can also be associated with cytoskeletal and membrane proteins (61). HSP70s have been found to be important during protein translation, folding, and transport in the cell, as well as in regulating protein aggregation and degradation (26, 59). In renal tubular epithelial cells, HSP70 associates with the sodium-dependent glucose transporter, increasing its expression at the apical membrane, resulting in enhanced glucose transport into the cell (30). Whether or not HSP70 interacts with facilitative glucose transporters or plays a role in the genesis and/or trafficking of GLUT4-containing vesicles is unknown. Alternatively, HSPs may serve as a sink for modified (e.g., O-GlcNAcylated) proteins that occur under various "stress" conditions (e.g., hyperglycemia). Interestingly, in rat liver extracts, members of the HSP70 family, including heat shock cognate protein 70 (HSC70), were found not only to be O-GlcNAc modified, but also to bind to, or serve as "lectins" for, other O-GlcNAc-modified proteins in both cytosolic and nuclear fractions (40). This observation may explain how the O-GlcNAc modification protects some proteins, e.g., Sp1, from degradation. A recent study implicates HSP70 in the regulation of protein kinase C (PKC) activity. HSP70 specifically binds to the unphosphorylated turn motif of the carboxy terminus of mature PKC (Thr⁶⁴² in PKC β II), thus stabilizing the protein, allowing rephosphorylation of the enzyme, and prolonging its half-life. This role of HSP70 appears to apply to all PKC isoforms [including PKC ζ and the other members of the AGC superfamily, e.g., Akt/PKB and PKA (17)]. We are currently testing the hypothesis that enhanced HSP70 O-GlcNAcylation may modify these interactions.

In the present study, we observed immunostaining with anti-O-GlcNAc of a "doublet" band at ~70 kDa from one-dimensional SDS-PAGE. This result, along with the observation that, on two-dimensional Western blots of HSP70 immunoprecipitates it appeared to migrate as several protein "spots," led us to speculate that we were detecting either more than one isoform of HSP70 or that the HSP70 that we isolated contained

several modifications, including O-GlcNAcylation. In skeletal muscle, four HSP70s are known to be present, including the glucose-regulated proteins GRP75 and GRP78, HSP72, and HSC70. After several MALDI spectra from tryptic digests were obtained and some of these tryptic peptides sequenced, our data indicate that the major HSP70 in our fractions is not a GRP (based on both tryptic fragment size and sequence) and matches most closely in all characteristics to rat HSP70.1/2 (SwissProt accession no. Q07439). The sequence AIAYGLDR that we sequenced from the 1,687.9-MW tryptic fragment is unique to HSP70.1/2; the similar fragment from HSC70 (accession no. NP077327) contains a COOH-terminal lysine rather than an arginine (AIAYGLDK). Therefore, it appears that the major HSP70 that we detected in our samples was HSP70.1/2, although we cannot rule out the presence of HSC70 or other unidentified HSP70s on the basis of the collective physical characteristics of the protein or the antibodies used in the study, as these are shared by or are reactive to all members of the HSP70 family.

The third protein that we identified as O-GlcNAcylated in L6 cells is the cytoskeletal protein α -tubulin. In adipocytes, integrity of the cytoskeletal network and motor proteins such as kinesin appear to be crucial for proper translocation of GLUT4-containing vesicles following insulin stimulation (13, 14, 19, 51). Microtubules are a critical component of the cytoskeletal network for cell motility and organelle and vesicle transport and are composed of tubulin α/β -heterodimers (50, 62). α -Tubulin was, in fact, found in purified preparations of GLUT4 vesicle membranes (19). A microtubule-associated protein, tau, found in neurons, is also modified by O-GlcNAc (1). The discovery that α -tubulin is O-GlcNAcylated and that this modification is enhanced under hyperglycemic conditions may be of significance, particularly if this modification affects microtubule dynamics and/or interactions with ligands. Tubulin is subject to numerous posttranslational modifications, including Ser/Thr phosphorylation (50), but the O-GlcNAc modification has not been previously reported. One of these modifications, enzymatic dephosphorylation, affects the interaction of tubulin with kinesin motors (56). In an in vitro microtubule-binding assay using 3T3-L1 adipocytes, Olson et al. (51) observed that GLUT4-containing vesicles and IRS-1 specifically bind to microtubules. Interactions between tubulin isoforms, including α -tubulin and PI 3-kinase, have been reported (31, 34, 35). Thus tubulin may play a role in regulating the intracellular trafficking of GLUT4 (13, 14, 19, 51) and of important mediators in the proximal insulin-signaling cascade (31, 34, 35, 51). Note, however, that the requirement for an intact microtubule network for insulin's stimulation of glucose transport and GLUT4 translocation has been questioned (45, 60).

Two recent articles strongly support a role for enhanced protein O-GlcNAcylation in glucose-induced insulin resistance (43, 63). When treated with a specific competitive inhibitor of O-GlcNAcase (21), 3T3-L1 adi-

pocytes manifest enhanced protein O-GlcNAc modification, insulin resistance of glucose transport, and Akt activation in the presence of normal IR/IRS-1/IRS-2 activation (63), thus mimicking the characteristics of glucose-induced insulin resistance in these cells (48). Transgenic mice with modest overexpression of O-GlcNAc transferase in skeletal muscle and adipocytes develop hyperinsulinemia, insulin resistance (assessed by euglycemic insulin clamp studies), and hyperleptinemia without changes in body weight, glycemia, or GLUT4 expression in muscle (43). We found that Sp1, HSP70, and α -tubulin are O-GlcNAcylated in L6 myotubes and that the O-GlcNAc modification on each of these proteins is markedly enhanced by exposure to high glucose and insulin. Further identification of glucose flux-responsive O-GlcNAcylated proteins may contribute to our understanding of glucose-induced insulin resistance in muscle. Whether or not the O-GlcNAc modification affects HSP70 and/or microtubular function and is implicated in insulin resistance of glucose transport in muscle warrants further investigation.

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