Constitutive Activation of GSK3 Down-regulates Glycogen Synthase Abundance and Glycogen Deposition in Rat Skeletal Muscle Cells*

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The effects of inhibition or constitutive activation of glycogen synthase kinase-3 (GSK3) on glycogen synthase (GS) activity, abundance, and glycogen deposition in L6 rat skeletal muscle cells were investigated. GS protein expression increased ∼5-fold during differentiation of L6 cells (comparing cells at the end of day 5 with those at the beginning of day 3). However, exposure of undifferentiated myoblasts (day 3) to 50 μM SB-415286, a GSK3 inhibitor, led to a significant elevation in GS protein that was not accompanied by changes in the abundance of GLUT4, another late differentiation marker. In contrast, stable expression of a constitutively active form of GSK3β (GSK3βS9A) led to a significant reduction (∼80%) in GS protein that was antagonized by SB-415286. Inhibition of GSK3 or expression of the constitutively active GSK3βS9A did not result in any detectable changes in GS mRNA abundance. However, the increase in GS protein in undifferentiated myoblasts or that seen following incubation of cells expressing GSK3βS9A with GSK3 inhibitors was blocked by cycloheximide suggesting that GSK3 influences GS abundance possibly via control of mRNA translation. Consistent with the reduction in GS protein, cells expressing GSK3βS9A were severely glycogen depleted as judged using a specific glycogen-staining antibody. Inhibiting GSK3 in wild-type or GSK3βS9A-expressing cells using SB-415286 resulted in an attendant activation of GS, but not that of glucose transport. However, GS activation alone was insufficient for stimulating glycogen deposition. Only when muscle cells were incubated simultaneously with insulin and SB-415286 or with lithium (which stimulates GS and glucose transport) was an increase in glycogen accretion observed. Our findings suggest that GSK3 activity is an important determinant of GS protein expression and that while glycogen deposition in muscle cells is inherently dependent upon the activity/expression of GS, glucose transport is a key rate-determining step in this process.

Glycogen synthase kinase-3 (GSK3)1 is a serine/threonine kinase that has been implicated in the control of numerous cellular responses including, for example, gene transcription, mRNA translation, intracellular signaling, and, of course, as its namesake suggests glycogen synthesis (for reviews see Refs. 1 and 2). The kinase is expressed in mammalian cells as two highly homologous isoforms, GSK3α and GSK3β, although a splice variant of the β-form has also recently been described (3). Although up to 40 putative substrates have been identified for GSK3, one of the best studied in vivo targets is glycogen synthase (GS) (1, 2). GS is regulated acutely by phosphorylation of at least nine different residues, four of which (sites 3a, 3b, 3c (collectively termed site 3), and site 4) are target sites for GSK3. Phosphorylation of GS by GSK3 requires that the enzyme be phosphorylated at a site four amino acids C-terminal to site 4 by a priming kinase (4). The primed phosphorylated residue on GS is thought to interact with a positively charged arginine residue (Arg36) located within the catalytic domain of GSK3 that allows docking and subsequent phosphorylation of GS on sites 3 and 4 by the kinase. Since GSK3 is constitutively active in unstimulated cells, GS is maintained in a phosphorylated and inactive state. However, in response to cell stimulation with insulin, GSK3 is itself inactivated by phosphorylation of an N-terminal serine residue (Ser21) in GSK3α and Ser9 in GSK3β) by a PI 3-kinase- and protein kinase B (PKB)-dependent mechanism (5). It has been proposed that the phosphorylated Ser21 residue folds back and interacts with the positively charged Arg36 residue creating a primed pseudosubstrate that occupies the positively charged pocket preventing the interaction of GSK3 with its normal physiological substrates (4). Under these circumstances, the inability of the kinase to engage its substrate alleviates the inhibitory input of GSK3 on GS, and the latter can then be dephosphorylated (and activated) by the glycogen-associated form of protein phosphatase 1 (PP1G).

Following insulin treatment the greatest decrease in bound phosphate on GS occurs at site 3 (6) suggesting that, in addition to control by allosteric regulators, the activation status of GSK3 is likely to be a major determinant of GS activity. If this proposition is correct, impaired inactivation or an increase in GSK3 activity/expression will have a profound effect on glycogen deposition and hence post-prandial glucose disposal in tissues such as skeletal muscle. Indeed, evidence exists in the

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1 The abbreviations used are: GSK3, glycogen synthase kinase-3; FBS, fetal bovine serum; GS, glycogen synthase; MAPK, mitogen-activated protein kinase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; RT, reverse transcriptase; β-Gal, β-galactosidase; PI, phosphatidylinositol; EV, empty vector.
literature showing that GSK3 expression is increased in adipose tissue of insulin-resistant mice (7) as well as in skeletal muscle of Type II diabetics where an inverse correlation with GS activity has also been reported (8). In the present study we have attempted to further define the role of GSK3 with respect to regulation of muscle GS and glycogen deposition by assessing the effects of manipulating GSK3 activity, through use of either selective GSK3 inhibitors or expression of a constitutively active form of GSK3β in which the N-terminal Ser9 residue has been mutated to an alanine (GSK3S9A), in L6 skeletal muscle cells. The expressed GSK3S9A is resistant to inactivation by insulin, but retains sensitivity to GSK3 inhibitors that act in an ATP-competitive manner. We show here that chronic inhibition of GSK3 in L6 myoblasts, which normally expresses a nominal amount of GS compared with differentiated myotubes, leads to an increase in GS protein abundance. Consistent with this finding we show that muscle cells expressing the active GSK3S9A mutant exhibit a marked reduction in GS protein, GS activity, and are substantially depleted in intramyocellular glycogen content. Exposing muscle cells expressing GSK3S9A to insulin does not prevent the loss in GS expression/activity whereas these effects were antagonized if cells were incubated with SB-415286 and SB-216763, two structurally unrelated maleimides that inhibit GSK3 (9). Our studies also reveal that whereas inhibition of GSK3 using these inhibitors promotes activation of GS to a level comparable to that seen in response to insulin that, unless accompanied by an increase in glucose transport, it alone is insufficient for stimulating glycogen accretion in muscle cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—α-Minimal essential medium (α-MEM), fetal bovine serum (FBS), anticytokinic/antibiotic were from Invitrogen, Life Technologies. Sterile-EDTA, insulin, UDP-glucose, cycloheximide, actinomycin D, lithium, and wortmannin were from Sigma-Aldrich. All primary and secondary antibodies were from Cell Signaling Technology (Beverly, MA) except antibodies against glycogen synthase (Chemicon International, Hampshire, UK), GSK3α (Division Signal Transduction Therapy, Dunde), GSK3β (Transduction Labs/BD Biosciences, San Jose, CA) and the γ-subunit of the Na/K-ATPase (Developmental Biology Hybridoma Laboratory, The University of Iowa, Iowa City, IA). Primes were synthesized by MWG Biotech (Milton Keynes, UK) and the Oligonucleotide Synthesis Laboratory (University of Dundee, UK). SB-415286 and SB-216763 were from Tocris (Bristol, UK).

**Cell Culture**—L6 muscle cells were cultured as described previously (13) (serum starvation 2% (v/v) FBS 1% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B) at 37 °C with 5% CO2. Cells were cultured in 10-cm dishes for lysis preparation and serum-starved for 5 h prior to addition of appropriate reagents for times and at concentrations indicated in figure legends. For cell studies involving amino acid deprivation, L6 cells were incubated in Earl’s Balanced Salt Solution (EBSS; 123 mM NaCl, 26 mM NaHCO3, 5 mM KCl, 1.8 mM CaCl2, 1 mM NaHPO4, 0.8 mM MgSO4, 5 mM glucose, pH 7.4) containing a physiological mixture of amino acids for 1 h followed by an additional 4 h either in the presence of amino acids (11).

**L6 Lysate Preparation**—L6 myoblasts were serum-starved as described above. Culture plates were washed three times with 0.9% (w/v) ice-cold saline. 200 μl of lysis buffer (50 mM Tris, pH 7.4, 0.27 M sucrose, 1 mM sodium orthovanadate pH 10, 1 mM EDTA, 1 mM EGTA, 10 mM Na β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1% (w/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 1 mM microcystin-LR, and protease inhibitors) was added. Cells were scraped and homogenized by passage through a 26-gauge syringe needle prior to centrifugation (13,000 × g, 3 °C for 5 min) and storage at −20 °C.

**Stable Transfection of L6 Muscle Cells with Constitutively Active GSK3β**—L6 cells were transfected using Lipofectamine reagent (Invitrogen, Life Technologies, Inc.) with pcDNA3 vector, which expresses GSK3 (S9A). L6 cells transfected with pGSMc were cultured as described earlier, but with the addition of 1 mg/ml of G418 sulfate to the media at all stages to select for transformed cells.

**Preparation of Adenoviruses and Infection of L6 Cells**—An adenovirus expressing GSK3α was generated using the AdEasy System as described previously (12) using plasmids that were a kind gift from Dr. B. Vogelstein (Howard Hughes Medical Institute, Baltimore, MD). The virally expressed GSK3α protein had an N-terminal deletion lacking serine 9 (rendering the expressed protein constitutively active) and the vector was bi-cistronic, also containing the gene encoding GFP. Control cells were infected with an adenovirus expressing β-galactosidase (β-Gal), which was a gift from Dr. Chris Newgard (University of Texas). The GSK3 and β-Gal adenoviruses were functionally titrated to assess the amount required for optimal infection efficiency in L6 cells was as determined by GFP imaging and β-galactosidase staining, respectively. L6 myoblasts were infected with equivalent quantities of adenovirus in serum-free medium (α-MEM) containing 5 mM d-glucose for 4 h at 37 °C. Cells were maintained in α-MEM containing 2% (v/v) FBS and incubated for 24 h at 37 °C prior to being serum-deprived for 5 h in the absence or presence of GSK3 inhibitors for times and at concentrations indicated in figure legends.

**Glycogen Synthase Assay**—Lysates from wild-type cells or those expressing GSK3S9A were prepared as described above. Glycogen synthase activity was assayed as described previously (13). Assay buffer (67 mM Tris pH 7.5, 5 mM dithiothreitol, 6.7 mM EDTA, 13 mg/ml glycogen, 1 μg/assay uridine diphospho-d-[6-3H]glucose) was added to 45 μl of lysate in the absence or presence of 20 μM glucose-6-phosphate. After a 30 min incubation at 37 °C the reaction was stopped by spotting the reaction mix onto 31ETFCHR Whatman filter paper and washed three times in 66% (v/v) ethanol for 20 min. Filters were washed in acetone and air-dried before incorporation of glucose from uridine diphospho-d-[6-3H]glucose into glycogen was quantitated by liquid scintillation counting. Glycogen synthase activity was expressed as a ratio of activity in the absence divided by that in the presence of its allosteric activator, glucose-6-phosphate.

**Glycogen Staining and Immunofluorescence**—L6 cells transfected with either empty pcDNA5 vector or pcDNA5 vector encoding constitutively active GSK3β cDNA (S9A) were grown on coverslips until confluent. Pretreated cells were fixed with 4% (w/v) paraformaldehyde, rehydrated with 2% (v/v) fish skin gelatin, and permeabilized with 0.2% Triton X-100 prior to incubation with 5% (v/v) donkey serum (Jackson Laboratories) to prevent nonspecific binding of secondary antibody and incubated overnight at 4 °C with an anti-glycogen antibody (1:50 in PBS containing 1% (v/v) donkey serum, (14)). Cells were incubated for 1 h at room temperature with 1:100 Alexa Fluor 594 conjugated donkey anti-mouse antibody (Molecular Probes, Netherlands) in PBS containing 1% (v/v) donkey serum prior to visualization using a Zeiss LSM 510 Meta confocal microscope.

**Immunoprecipitation**—100 μl of protein G-Sepharose beads were washed three times in PBS (150 mM NaCl, 2.68 mM KCl, 12 mM NaHPO4, 1.77 mM KH PO4, pH 7.4) and incubated with either c-Myc (1:1000) or GSK-3β (5 μg), for 4 h at 4 °C on an orbital platform shaker. Bead/antibody mix were washed three times in PBS and incubated with 50 μl of rabbit anti mouse IgG (1:1000) or HRP-anti mouse IgG (1:1000) as appropriate. Protein signals were visualized using enhanced chemiluminescence by exposure to Kodak autoradiographic film.

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buffer. GSK3 was immunoprecipitated from 100 μg of cell lysate using isoform-specific antibodies to GSK3α or GSK3β or an anti-Myc antibody and incubated with or without 25 milliunits/ml FPP2A, prior to assaying kinase activity using phospho-GS peptide-1 as substrate.

Reverse Transcriptase (RT)-PCR—Oligonucleotide primer pairs were synthesized (MWG Biotech AG, Ebersberg, Germany) to match bp 729–752 (GS sense, 5′-ATAGACAAAGAGGCGGGAGGAGG-3′) and bp 1133–1156 (GS antisense, 5′-TTGGCAGGCTAGATGAAAAACACT-3′) of GS sequence and bp 826–846 (β-actin sense, 5′-AGAGCCAATCCACACAGA-3′) and bp 10969–1113 (β-actin antisense, 5′-ATATCGAGCGATGGCCGTTTC-3′) of rat muscle β-actin sequence. Total RNA was isolated from L6 myoblasts and GSK39A cells using TRIzol® reagent as per manufacturer’s instructions (Invitrogen, Life Technologies). RT-PCR was performed using the One-Step RT-PCR kit (Novagen, San Diego, CA). The reaction mix (1 μg of RNA, 2.5 mM dNTP, 25 mM Mn, 5× reaction buffer, 20 units of RNase inhibitor, 250 μM sense primer of GS and of β-actin, 250 μM antisense primer of GS and of β-actin to a final volume of 12 μl with RNase/DNase-free water) was subjected to a manual hot start (60 °C for 3–5 min), 5 units of rTth polymerase were added, and reverse transcription performed at 60 °C for 30 min. The following conditions were used for PCR amplification: PCR activation was initiated by 2 min at 94 °C, denaturing occurred at 94 °C for 1 min and annealing/extension was repeated for 24 cycles, final extension was performed for 7 min at 60 °C. Products were resolved on a 1% (w/v) agarose gel in TBE (89 mM Tris, 89 mM boric acid, 2 mM Na2EDTA) with 0.5 μg of ethidium bromide and visualized using a UV transilluminator.

RNA Extraction and RT for Real-Time PCR—Total RNA was isolated from L6 myoblasts and GSK39A cells using TRIzol® reagent as per manufacturer’s instructions (Invitrogen, Life Technologies). Samples were further purified using RNaseasy Mini kits (Qiagen). RT was performed using the iScript cDNA synthesis kit (Bio-Rad). The reaction mix (500 ng of RNA and 5× reaction mixture to a final volume of 20 μl with RNase/DNase free water) was subjected to the following RT conditions: 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C.

Real-Time PCR—Oligonucleotide primer pairs were designed by Beacon Designer to match bp 485–505 (GS sense, 5′-TGGTCTCT-GGGTGAGTGCTCTC-3′) and bp 607–627 (GS antisense, 5′-GTTGGCAGGCTAGATGAAAAACACT-3′) of muscle glycogen synthase (GS) sequence and bp 5584–5603 (18 S sense, 5′-GTAACCCGTTAGACCCAT-3′) and bp 5715–5734 (18 S antisense, 5′-CCATCACAATGCGTAACTGCG-3′) of mouse 18 S sequence and synthesized by the Oligonucleotide Synthesis Laboratory. 18 S rRNA was used as a reference for normalization as described previously (15). Real-time PCR was performed using a Bio-Rad iCycler (Bio-Rad) with SYBR green fluorophore. 1 μl of previously reverse-transcribed cDNA template was mixed with 10 μl of IQSYBR Green Supermix (Bio-Rad), 400 nM of sense primer of GS and of 18 S, 400 nM of antisense primer of GS and of 18 S, to a final volume of 20 μl with RNase/DNase-free water.

Protocols for each primer set were optimized using 7 serial 10× dilutions of template cDNA. The following PCR protocol was used: 95 °C for 3 min, 95 °C for 20 s and 61 °C for 45 s repeated for 40 cycles, 95 °C for 1 min, 55 °C for 1 min, 55 °C for 10 s with a 0.5 °C increase in temperature each cycle for 80 cycles and 25 °C for 5.5 min. All reactions were carried out to n = 3.

Statistical Analyses—For multiple comparisons statistical analysis was performed using one way analysis of variance (ANOVA) followed by a Newman Keuls post test. Data analysis was performed using GraphPad Prism software and considered statistically significant at p values <0.05.

RESULTS

GS Expression in L6 Myoblasts and Myotubes and the Effects of GSK3 Inhibition—Preconfluent L6 myoblasts rapidly divide and align in culture and upon confluence, spontaneously fuse to form multinucleated myotubes. During this process of differentiation, muscle cells exhibit increased insulin binding (16) and GLUT4 expression (17) indicative of a transition to an increased insulin-responsive state. To test whether GS expression also increases during myogenesis we immunoblotted lysates prepared from preconfluent (early day 3) and confluent (late day 5) L6 muscle cells with anti-GS antibodies. Fig. 1A shows that GS expression was ~5-fold greater in lysates prepared from cells harvested at the end of day 5 compared with those lysed at the beginning of day 3 of the differentiation process. Consistent with previous observations (17), analysis of GLUT4 abundance in the same cell lysates revealed a near 4-fold increase in GLUT4 expression in lysates from confluent (day 5) cells. The observed increase in GS and GLUT4 are likely to be a differentiation-linked phenomenon given that no parallel changes in the expression of the α1-subunit of the Na+/K+ pump to establish equal loading of cell lysate protein. C, RNA isolated from myoblasts (day 3) that had been pre-treated with 50 μM SB-415286 for 24 h was subjected to semiquantitative RT-PCR as described under “Experimental Procedures.” Primers against glycogen synthase and β-actin were used, and amplified PCR products of 428 and 288 bp respectively were detected on a 1% (w/v) agarose gel. The blots are representative of at least three separate experiments.

Unlike GS, we have shown previously that expression of GSK3α and GSK3β does not alter significantly during differentiation of L6 cells (13). However, on the basis that preconfluent myoblasts are less sensitive and responsive to insulin than those at a later stage of differentiation, GSK3 activity is likely to be higher during the early stages of the myogenic growth program. If this proposition is correct then it is plausible that high cellular GSK3 activity may act to suppress GS expression during early differentiation. To test this proposition we incubated preconfluent (early day 3) L6 myoblasts in the absence and presence of SB-415286 (an ATP competitive GSK3 inhibitor). Previous studies from our lab have shown that this inhibitor causes a profound inhibition of GSK3 as reflected by complete loss in GSK3-mediated phosphorylation of site 3 on GS (13). Fig. 1B shows that a 24-h incubation with the inhibitor led...
to a significant increase in the expression of GS protein in L6 myoblasts. Under these circumstances we did not observe any increases in the abundance of GLUT4 (a late differentiation marker, Fig. 1A) or of the α1- Na+/K-ATPase subunit. Inhibition of GSK3 was associated with the appearance of a GS protein that displayed a distinct shift in gel mobility to a faster migrating band, this finding is fully consistent with reduced phosphorylation of GS on sites 3 and 4 that would be expected as a result of GSK3 inhibition. Increased GS abundance was not observed when myoblasts were incubated with SB-415286 for periods less than 4 h (Fig. 1B), suggesting that GSK3 may regulate cellular GS abundance via transcriotional or transla- tional control. Analysis of GS mRNA however, revealed no detectable increase in GS gene transcription in SB-415286 treated cells (Fig. 1C).

GSK3β Overexpression in L6 Cells—To further help define the role of GSK3 in the regulation of GS and its contribution to glycogen deposition in L6 muscle cells we generated muscle cells stably expressing a constitutively active (S9A) GSK3β mutant harboring a c-Myc tag. Cells expressing the empty vector (EV) were used as control. Immunoblotting of GSK3β immunoprecipitates with a c-Myc antibody confirmed the expression of the GSK3S9A (Fig. 2A), which was further verified by performing the reciprocal analysis (i.e. screening c-Myc immunoprecipitates with an anti-GSK3β antibody). The total expression levels of GSK3β in myoblasts (day 3 and day 5) were compared between cells transfected with the EV or the GSK3S9A using a GSK3β antibody. No significant differences in GSK3β abundance was observed in myoblasts transfected with the EV after 2 and 5 days of differentiation. However, myo- blasts expressing the S9A construct displayed a modest, but significant overexpression of GSK3β expression at day 3, which was increased by over 2-fold by day 5 of differentiation (Fig. 2B). Over this period, stable transfection of GSK3S9A had no impact on the cellular expression of GSK3α in L6 cells.

GSK3 Activity in Muscle Cells Overexpressing GSK3S9A—Having established that GSK3S9A was significantly overexpressed in day 5 L6-myoblasts we subsequently assessed the effects of this constitutively active kinase on GSK3 activity in response to an insulin challenge. Fig. 3A shows that insulin induces a significant inactivation of GSK3α (40%), but that this was lost by prior treatment of muscle cells with the PI 3-kinase inhibitor, wortmannin (WM). This finding is fully consistent with the view that the hormonal inactivation of GSK3 isoforms is mediated in a PI 3-kinase-dependent manner (5). In contrast, however, insulin failed to inactivate GSK3β in these cells irrespective of whether the kinase had been precipitated for assay using an antibody to the c-Myc tag or GSK3β itself. This observation implies that the Myc-tagged kinase is constitutively active and by virtue of its overexpression is dominant over the endogenous GSK3β whose inactivation we could.
not detect by this method. To demonstrate that the endogenous GSK3 isoforms are being phosphorylated and inactivated we utilized phosphospecific antibodies against Ser21/9 of GSK3. Fig. 3B shows that insulin induces GSK3 phosphorylation of both GSK3α and β in EV and GSK3S9A-expressing cells and that this was abolished by prior exposure of L6 cells to wortmannin.

**Effects of Constitutive GSK3β Activation on GS and β-Catenin Protein Expression**—To determine whether a constitutive increase in GSK3 activity negatively regulates cellular GS abundance we probed whole cell lysates prepared from day 5 EV- and GSK3S9A-expressing muscle cells. GS was detected in EV-expressing myoblasts and inhibition of GSK3 (with SB-415286 for 4 h) did not modify total GS abundance as had been observed in undifferentiated day 3 myoblasts (Fig. 1). However, as with untransfected day 3 myoblasts, GSK3 inhibition did induce a slight shift in the electrophoretic mobility of GS in EV-containing cells (Fig. 4A, compare lanes 1 and 2). Strikingly, however, when GS abundance was assessed in day 5 GSK3S9A-expressing muscle cells we observed a reduction by ~80% compared with those expressing the EV (Fig. 4A, compare lanes 1 and 3). This loss in GS expression is likely to be attributable to the much higher GSK3 activity present in GSK3S9A cells as suppressing the activity of the constitutively active and endogenous kinase using SB-415286 or SB-216763, a structurally unrelated GSK3 inhibitor (9), induced a time-dependent increase in GS abundance (Fig. 4A, compare lane 3 with lanes 4, 5, and 6). In this experiment, the abundance of p42/p44 MAP kinases served to verify equivalent loading of protein samples on the gel.

To help further consolidate the finding that GS abundance is reduced in cells expressing a constitutive increase in GSK3 activity we utilized a transient adenoviral transfection approach. On day 5 of differentiation, confluent myoblasts were infected with adenoviral vectors encoding either β-Gal (control) or an N-terminal truncated GSK3α (i.e. ΔN-term-GSK3α) lacking Ser21 that renders the expressed kinase insensitive to insulin. This construct also contains cDNA encoding GSP. Fig. 4B shows that 24-h postinfection, cells exposed to the control viral vector displayed greater GS abundance than those infected with virus encoding the ΔN-term-GSK3α. Consistent with earlier findings, treatment of muscle cells with SB-415286 not only induced an electrophoretic mobility shift in GS, but also antagonized the loss in GS abundance induced by expressing the ΔN-term-GSK3α construct. Confirmation that ΔN-term-GSK3α was indeed expressed in infected muscle cells was gained by immunoblotting cell lysates with an anti-GSK3 antibody that detected the truncated kinase as a slightly faster migrating band (Fig. 4B) and by fluorescence imaging of the expressed GFP contained within the viral vector (Fig. 4C).

In an attempt to determine the underlying mechanism by which SB-415286 antagonizes the suppression in GS protein content in cells expressing a constitutive increase in GSK3 activity we assessed whether GS mRNA was increased in response to treatment with the inhibitor. However, as observed with untransfected day 3 L6 myoblasts (Fig. 1C), the increase in cellular GS content seen following incubation with the GSK3 inhibitor could not be attributed to enhanced GS gene transcription (Fig. 5A). It is plausible that semiquantitative RT-PCR may not be sufficiently sensitive to detect changes in GS mRNA. To exclude this possibility we subsequently performed quantitative real-time PCR. Fig. 5B shows that the level of GS mRNA in both L6 myoblasts (day 3) and muscle cells expressing the GSK3S9A was not significantly altered when expressed and normalized relative to 18 S rRNA. The suggestion that changes in GS mRNA are unlikely to account for the increase in GS protein abundance following inhibition of GSK3 was further strengthened by the observation that GS protein content could be elevated by SB-415286 in cells treated with the transcriptional inhibitor, actinomycin D (Fig. 5C). The efficacy of the inhibitor was confirmed by its ability to prevent the increase in the expression of the SNAT2 System A transporter when cells were subjected to a 4-h amino acid deprivation (Fig. 5C). System A expression is well documented to increase in response to amino acid withdrawal by a transcriptionally dependent mechanism (19).

We subsequently investigated whether the up-regulation of GS protein could be attributed to changes in mRNA translation. Fig. 6A shows that the increase in GS protein brought about by SB-415286 in cells expressing the constitutively active GSK3S9A was ablated when cells were simultaneously exposed to SB-415286 and cycloheximide, an inhibitor of protein synthesis. These findings indicate that in cells expressing the GSK3S9A, a constitutive increase in GSK3 activity may act to suppress synthesis of proteins such as GS. GSK3 is known to suppress mRNA translation via phosphorylation of the epsilon subunit of eIF2B, a guanine nucleotide exchange factor that
plays a critical role in translation initiation. Consistent with this possibility, eIF2Be was phosphorylated in L6 cells expressing GSK3βα/S9A and its dephosphorylation was found to be impaired in response to insulin, but not to SB-415286 (Fig. 6B). One expectation of increased eIF2Be phosphorylation would be a suppression of global protein synthesis. However, analysis of total protein in cells expressing the empty pSG5 vector or GSK3βα/S9A or on day 3 revealed only a marginal reduction in the latter (empty vector, 0.395 ± 0.2 mg; GSK3βα/S9A, 0.328 ± 0.2 mg, values are mean ± S.E. from three separate experiments with a starting seeding density of 10^5 cells/35-mm dish). To establish whether the increase in GS protein that we had observed was due to increased GSK3β expression and upon phosphorylation of eIF2Be in L6 muscle cells. L6 muscle cells expressing GSK3βα/S9A (S9A) were incubated in the absence or presence of either 100 nM insulin for 10 min or 50 μM SB-415286 for 24 h prior to RNA isolation and (A) semi-quantitative RT-PCR to assess GS and actin mRNA abundance as described under “Experimental Procedures.” The relative abundance of GS and actin mRNA was expressed as a ratio, or (B) real-time PCR to assess GS mRNA relative to 18 S rRNA abundance as described under “Experimental Procedures.” Values represent mean ± S.E. of three separate experiments. C, L6 muscle cells expressing S9A were pretreated with 5 μg/ml actinomycin D (Act D) for 5 h either in the presence of absence of 50 μM SB-415286 for the final 4 h prior to lysing and immunoblotting with antibodies to GS. L6 myoblasts (day 3) were incubated with 5 μg/ml actinomycin D (Act D) for 5 h in EBSS containing a physiological mix of amino acids for 1 h followed by an additional 4 h in EBSS in the presence or absence of amino acids. Cells were lysed and immunoblotted with antibodies to the system A amino acid transporter, SNAT2. Equal loading of cell lysate protein was determined by probing with an antibody to p42/44 MAPK. The blots shown are representative of a minimum of three separate experiments.

A particular concern that has been raised with respect to use of GSK3 inhibitors is that long term inhibition of GSK3 may increase the risk of oncogenesis as a result of increased accumulation of β-catenin, a component of the Wnt signaling pathway (20). In contrast, constitutive activation of GSK3β would be expected to promote phosphorylation and proteosomal degradation of β-catenin. However, unlike the effects observed on GS protein, neither acute (1 h) nor sustained (24 h) inhibition of GSK3β with SB-415286 or expression of a constitutively active GSK3β in L6 cells had any detectable effect on β-catenin abundance in this in vitro culture system (Fig. 7).
glycogen content is directly influenced by manipulating GSK3 activity we utilized a monoclonal antibody to visualize glycogen staining in muscle cells expressing the EV or GSK3S9A. Fig. 9A shows punctate Alexa Fluor 594 staining of glycogen in unstimulated day 5 L6 cells. In contrast, Fig. 9B shows that GSK3S9A expressing cells maintained in culture over the same period were severely glycogen-depleted. Stimulating muscle cells with insulin for 30 min significantly enhanced the intensity of the Alexa Fluor 594 emission signal from EV cells consistent with increased synthesis and deposition of glycogen in these cells (Fig. 9C). No such increase was observed in muscle cells expressing GSK3S9A (Fig. 9D).

When EV or GSK3S9A expressants were preincubated with SB-415286 for 4 h (in order to both inhibit GSK3 and induce GS protein expression, respectively) the extent of glycogen staining was not substantially greater than that observed in unstimulated cells (compare Fig. 9, A with E and B with F). However, when muscle cells were incubated with insulin following a 4-h preincubation period with SB-415286, glycogen deposition was significantly greater in both EV and GSK3S9A expressants compared with treatment of these respective cell groups with either stimulus alone (Fig. 9, G and H). Intriguingly, cell incubation with lithium (Li, a non selective GSK3 inhibitor) led to an increase in glycogen staining in both EV and GSK3S9A muscle cells (Fig. 9, I and J). The finding that Li, but not SB-415286, promotes glycogen accretion in cells expressing the GSK3S9A implies that Li must...
affect additional cellular targets that help support an increase in glycogen synthesis.

**DISCUSSION**

In addition to control by allosteric regulators the phosphorylation status of glycogen synthase (GS) is a key determinant of its activity. Several kinases are known to phosphorylate GS, but it is well established that GSK3 represents one of the principal GS kinases that helps maintain the enzyme in a repressed state in unstimulated cells (21). This repression can be rapidly reversed upon cell stimulation with insulin, which promotes the inactivation of GSK3 and the dephosphorylation of GS by PP1G. While it is generally accepted that GSK3 plays a key suppressive role in the acute regulation of GS function very little information exists on whether the activity of the kinase also influences the cellular expression of GS protein in tissues such as skeletal muscle. Thus a major objective of the present study was to assess the effects of manipulating GSK3 activity, using either small molecule inhibitors or by expression of a constitutively active form of the kinase, on GS abundance and glycogen deposition in rat skeletal muscle cells. Three major novel findings have emerged from the present study. First, GS expression is up-regulated during differentiation of L6 myoblasts to myotubes, but can be induced significantly during early differentiation by inhibiting the catalytic activity of GSK3. Second, transient or stable overexpression of a constitutively active form of GSK3 in confluent L6 muscle cells promotes a reduction in GS abundance that can be antagonized using GSK3 inhibitors. Furthermore, consistent with the reduced GS abundance in muscle cells expressing the constitutively active kinase such cells are severely glycogen depleted. Finally, we demonstrate that in our experimental system sustained inhibition of GSK3 or expression of the constitutively active kinase neither acts to suppress or elevate cellular β-catenin levels.

While it has previously been demonstrated that GS activity is suppressed in HEK293 cells (22) and 3T3-L1 adipocytes (23) transiently overexpressing constitutively active GSK3β (S9A), the finding that long term inhibition or activation of GSK3 alters the cellular abundance of GS protein has not, to our knowledge, been reported before. There is considerable evidence in the literature showing that GSK3 can regulate gene expression via control of numerous transcription factors such as AP-1, CREB, NFAT, Myc, C/EBPα, and β-catenin (12, 24, 25). However, our studies reveal that up-regulation of GS in L6 myoblasts or muscle cells stably expressing the GSK3S9A mutant following incubation with SB-415286 occurs without any detectable change in GS mRNA abundance, at least over the 24-h period of incubation with the inhibitor. The increase in GS protein observed under these circumstances is nevertheless sensitive to cycloheximide suggesting that GSK3 is likely to exert its control at the level of translation. This proposition is supported by the finding that GSK3 can suppress protein synthesis via phosphorylation of the guanine nucleotide exchange factor eIF2B, which links global regulation of protein synthesis to the PI 3-kinase/PKB signaling pathway (26). Muscle cells expressing GSK3S9A exhibit phosphorylation of eIF2B and Ser423/425 and, as such, this was refractory to dephosphorylation by insulin, a finding that is fully consistent with the inability of the hormone to suppress the constitutive input from the mutated GS3K (S9A) kinase. However, since GSK3S9A remains sensitive to SB-415286 the inhibitor overrides this input allowing for the dephosphorylation of eIF2B and thereby facilitating an increase in translation initiation. While this mechanistic scenario is attractive the loss in GS protein seen in cells expressing the GSK3S9A is proportionately greater than the overall suppression in protein synthesis (as judged on the basis of total cell protein which was reduced by up to 20% in such cells) suggesting that the loss in cellular GS is unlikely to be explained by just a reduction in the global rate of protein synthesis. Another plausible explanation that may account for the reduction in muscle cell GS content is enhanced proteosomal degradation. A number of cellular GSK3 targets such as β-catenin, p21Waf1, Rap1, and cyclin D1 are directed for proteosomal lysis following phosphorylation (27–30). However, in separate experiments we have observed that suppressing proteosomal-dependant protein degradation, using MG132, does not rescue the loss in cellular GS nor does it augment the effects of SB-415286 on GS protein levels in L6 cells expressing the constitutively active form of GSK3 (data not shown). The findings therefore imply that in addition to acutely regulating GS phosphorylation and activity, GSK3 also conveys a tonic stimulus that appears to be important in the translation of GS mRNA, at least in this experimental system. The precise mechanism by which this occurs remains to be defined and serves as a focus for future study.

The relative importance of glucose transport and GS in controlling the rate of muscle glycogen deposition has been a long standing debate. The finding that muscle cells expressing GSK3S9A exhibit a substantial reduction in GS and glycogen content, yet display no significant changes in basal or insulin-stimulated glucose transport, supports a pivotal role for GS in the control of glycogen deposition. These observations are consistent with very recent work showing that transgenic mice overexpressing GSK3β exhibit reduced muscle GS activity (total GS protein was not assessed) and are depleted significantly in their muscle glycogen content by nearly 60% (31). Nevertheless, our data also provides strong support for the view that glucose transport is a major determinant of glycogen deposition since activation of GS by SB-415286 (or SB-216763), to a level comparable to that elicited by insulin, failed to promote glycogen accretion in muscle cells as judged using a specific glycogen staining antibody (compare panels E and F with A–D of Fig. 9). Although some GSK3 inhibitors have been reported to stimulate glucose transport in skeletal muscle of insulin-resistant Zucker rats and humans (32, 33), neither of two maleimide inhibitors used in this study exert any stimulatory effect on glucose uptake in L6 muscle cells. Consequently, activation of GS in L6 muscle cells (or indeed recovery and activation of the enzyme in cells expressing GSK3S9A) appears insufficient for stimulating glycogen accumulation without having an accompanying increase in glucose transport. This suggestion is strengthened by the finding that simultaneous exposure of muscle cells to SB-415286 and insulin, or indeed to lithium alone (which is capable of stimulating both glycogen synthase (as a result of GSK3 inhibition) and glucose transport (13)), leads to an increase in glycogen synthesis based on the enhanced intensity of glycogen staining (e.g. compare panels G–J with panels A–F in Fig. 9). Our results would indicate that while glycogen deposition cannot occur without GS, glucose transport is rate-limiting for glycogen synthesis. This proposition is supported by studies in transgenic mice in which it has been shown that control of glycogen deposition is shared between GS and glucose transport (34). Overexpression of GS in mouse skeletal muscle enhances intramuscular glycogen deposition without any significant increase in glucose transport implying that glucose can be “pulled” into glycogen by an increase in total GS activity. However, despite the increase in muscle GS, glycogen accumulation is ultimately restricted because of a substantial reduction in intramuscular UDP-glucose suggesting that substrate (glucose) supply becomes rate-limiting and that there is, therefore, a strict requirement to also “push” glucose into glycogen via activation of glucose transport (34).
An increase in GSK3 activity has been implicated in the pathogenesis of insulin resistance in diabetic- and obesity-prone rodents as well as diabetic subjects (7, 8) and overexpression of GSK3 in either cultured cells (35) or mice (31) has been shown to antagonize insulin signaling by promoting a loss of IRS-1. Such observations would imply that selective small molecule inhibitors of GSK3 may be of therapeutic value in the treatment of diabetes and indeed several recent studies have reported that suppressing GSK3 activity has “insulin-like” effects. GSK3 inhibitors have been shown, for example, to lower blood glucose, and stimulate both glucose transport and glycogen synthesis in insulin-resistant rats (32, 36), increase IRS-1 expression and stimulate glucose uptake in human muscle (33) and activate glycogen synthase independently of insulin in cell-based systems (9, 13). However, while the potential of GSK3 inhibitors may be of value in the treatment of insulin resistance, such inhibitors may have undesirable effects upon control of other important cell functions that rely upon GSK3 activity. GSK3 for example, plays a critical role in the Wnt signaling pathway where it is responsible for phosphorylating β-catenin, a downstream component of the Wnt pathway (37). Phosphorylation of β-catenin requires that GSK3 be held in a protein complex consisting of the scaffold protein axin, APC (adenomatous polyposis coli) and β-catenin. In unstimulated cells, casein kinase 1 is recruited to this complex and phosphorylates β-catenin on Ser45, which is recognized by GSK3 as a primed site that then allows it to phosphorylate β-catenin on Thr41, Ser37, and Ser33 (20). Phosphorylated β-catenin is then directed for proteosomal degradation. However, in response to Wnt, additional proteins such as dishevelled and FRAT (frequently rearranged in advanced T-cell lymphomas) are recruited to the axin/PCSK3 complex resulting in the displacement of axin and a loss in the GSK3-directed phosphorylation of β-catenin. Under these circumstances non-phosphorylated β-catenin accumulates and can translocate to the nucleus where it can form complexes with TCF/LEFs and transactivate the expression of numerous genes including oncogenic target genes, such as c-Myc, cyclin D1, and c-Jun (38).

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Constitutive Activation of GSK3 Down-regulates Glycogen Synthase Abundance and Glycogen Deposition in Rat Skeletal Muscle Cells
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