O-Linked N-Acetylgalactosaminyltransferase Is Involved in the Ca\(^{2+}\) Activation Properties of Rat Skeletal Muscle* 

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O-Linked N-acetylgalactosaminylation termed O-GlcNAc is a dynamic cytosolic and nuclear glycosylation that is dependent both on glucose flow through the hexosamine biosynthesis pathway and on phosphorylation because of the existence of a balance between phosphorylation and O-GlcNAc. This glycosylation is a ubiquitous post-translational modification, which probably plays an important role in many aspects of protein functions. We have previously reported that, in skeletal muscle, proteins of the glycolytic pathway, energetic metabolism, and contractile proteins were O-GlcNAc-modified and that O-GlcNAc variations could control the muscle protein homeostasis and be implicated in the regulation of muscular atrophy.

In this paper, we report O-N-acetylgalactosaminylation of a number of key contractile proteins (i.e. myosin heavy and light chains and actin), which suggests that this glycosylation could be involved in skeletal muscle contraction. Moreover, our results showed that incubation of skeletal muscle skinned fibers in N-acetyl-O-glucosamine, in a concentration solution known to inhibit O-GlcNAc-dependent interactions, induced a decrease in calcium sensitivity and affinity of muscular fibers, whereas the cooperativity of the thin filament proteins was not modified. Thus, our results suggest that O-GlcNAc is involved in contractile protein interactions and could thereby modulate muscle contraction.

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5 The abbreviations used are: O-GlcNAc, O-linked N-acetylgalactosaminylation; MHC, myosin heavy chain; MLC, myosin light chain; WGA, wheat germ agglutinin; EDL, extensor digitorum longus; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid.

and threonine hydroxyl group by a β-linkage (1). Because of the existence of the UDP-GlcNAc-peptide-β-GlcNAc transferase, which transfers the monosaccharide into proteins (2, 3), and the N-acetyl-β-glucosaminidase, which removes it (4), O-GlcNAc is more similar to phosphorylation than classical glycosylation. The half-life of the monosaccharide is shorter than the half-life of the protein backbone (5), indicating that the O-GlcNAc cycle could rapidly respond to cellular signals (6). All of the known O-GlcNAc proteins described to date are also phosphoproteins (7) (for review, see Ref. 8). Modifications by O-GlcNAc or phosphorylation could occur on the same site (9) or at neighboring sites (10); this competition between O-GlcNAc and phosphorylation is called the “ Yin-Yang” process.

O-GlcNAc has been described to play a role in various cell functions: in nuclear transport (11–13); in protein degradation, with a reversible inhibition of the proteasome itself (14) and a protection of the modified protein against proteasomal degradation (9, 15, 16); and in regulation of protein expression, with the regulation of transcription (10, 17–19) and translation (20). The involvement of O-GlcNAc in protein-protein interactions has been described in different biological systems. Indeed, many proteins playing a key role in organization and assembly of cytoskeleton are O-GlcNAc-modified, including cytokertains 8, 13, and 18 (21, 22), H, L, and M neurofilaments (23, 24), microtubule-associated proteins MAP1, -2, and -4 (25), crystallin (5, 26), synapsin 1 (27, 28), and Tau protein (29). Four sites of O-GlcNAc have been described on neurofilaments (23), these sites being localized on the head filament, which is involved in the polymerization of filament (30). Moreover, the interaction between translation initiation factor eIF-2 and p67 protein is also dependent on O-GlcNAc (20), the deglycosylation of p67 producing the dissociation of the complex.

Key proteins involved in the skeletal muscle metabolism and in the contractile process have been recently identified as being O-GlcNAc-modified (31). The implication of O-GlcNAc in muscle physiology remains undetermined even if recent results suggest that O-GlcNAc variations could control the muscle protein homeostasis and are involved in the regulation of muscular atrophy (32) as well as in the glucose metabolism (33).

The functional significance of the O-GlcNAc modification of myosin heavy chain (MHC) could be its implication in protein-protein interaction, resulting in polymerization of the thick filament, or its involvement in the development of the muscle contraction by interacting with other contractile proteins. Therefore, the purpose of the present study was (i) to define...
whether other contractile proteins could be O-GlcNAc-modified and (ii) to determine a potential role of O-GlcNAc post-translational modification in skeletal muscle contraction.

EXPERIMENTAL PROCEDURES

Biochemicals—Agarose-immobilized wheat germ agglutinin (WGA), GlcNAc-immobilized beads, and all chemical reagents were purchased from Sigma; sequencing grade modified trypsin was from Promega (Madison, WI); the mixture of antiproteases was from Amersham Biosciences; Zip-Tip 

C18 pipette tips were from Millipore (Bedford, MA); Vivasin concentrators were from Vivascience (Hannover, Germany); the nitrocellulose sheet was from Advantec MFS (Pleasanton, CA); and the MicroBCA protein assay reagent kit was from Pierce.

Animals and Muscle Preparation—Experiments were carried out on skeletal muscles of adult male Wistar rats. The experiments as well as the maintenance conditions of the animals received authorization from the Ministry of Agriculture and the Ministry of Education (Veterinary Service of Health and Animal Protection, authorization 03805).

Soleus (a typical slow postural muscle), extensor digitorum longus (EDL, a typical fast muscle), and gastrocnemius (a mixed composition of fibers) muscles were freshly removed from male Wistar rats (n = 10) anesthetized with an intraperitoneal injection of pentobarbital sodium (3 mg·kg⁻¹), quickly frozen, and pulverized in liquid nitrogen. All samples were kept at −80 °C until analyzed.

Contractile Protein Preparation—Muscle powder was resuspended in a 5 mM EDTA solution, pH 7.0, containing antiproteases. Sample was homogenized for 5 min and then centrifuged at 4 °C for 10 min at 13,000 rpm. Pellet was washed twice using a 50 mM KCl solution containing anti-proteases and centrifuged at 4 °C for 10 min at 13,000 rpm.

Purification of O-GlcNAc-modified Proteins—The pellet of contractile proteins described above was resuspended in the binding buffer (20 mM Tris/HCl, 200 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, antiproteases, pH 7.8), homogenized by ultrasonic waves (Cell Disruptor B-30). Protein quantification was performed using the MicroBCA protein assay. Isolation of O-GlcNAc proteins was obtained by passing contractile proteins through a WGA-immobilized column. For each experiment, 500 µg of contractile proteins were used. The column was washed with 50 column volumes of binding buffer, followed by washing with 10 column volumes of binding buffer containing 0.2 M GalNAc. Elution was finally performed with 20 column volumes of 0.2 M GlcNAc in the same buffer. Fractions were desalted and concentrated using centrifugation on a Vivasin concentrator. Samples were resuspended in Laemmli buffer just prior to their electrophoretic analysis. To determine the glycosylation of the muscle biopsies, a similar protocol was just prior to their electrophoretic analysis. To determine the glycosylation of the muscle biopsies, a similar protocol was used. The focusing potential was set at −220 V, and the declustering potential varied between −15 and −50 V. For the recording of conventional mass spectra, TOF data were acquired by accumulation of 10 multiple channel acquisition scans over mass ranges of m/z 500–2000. In the collision-induced dissociation MS/MS analyses, multiple charged ions were fragmented using nitrogen as a collision gas (4 × 10⁻³ torr, 1 torr = 133.3 pascals) with a collision energy between −40 and −55 eV, according to the sample, to obtain optimal fragmentation. The collision-induced dissociation spectra were recorded on the TOF analyzer over a range of m/z 150–2000. All signals were resolved monoisotopically.

Confirmation of the Glycosylation Type of Contractile Proteins by Immunoblotting—Contractile proteins (30 µg) of soleus and EDL muscles were separated using a linear SDS-PAGE gradient gel (10–20%) and were electroblotted on nitro-
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cellulose membrane. Membranes were saturated for 45 min with 5% bovine serum albumin in TBS-Tween buffer (50 mM Tris/HCl, 150 mM NaCl, 0.05% Tween, pH 8.0). RL2 anti-O-GlcNAc monoclonal antibodies were incubated overnight at 4 °C at a dilution of 1:1000. Three washes of 10 min each were performed with TBS-Tween. Anti-mouse horseradish peroxidase-labeled secondary antibodies were used at a dilution of 1:10,000 for 1 h at room temperature. After three washes, the detection was carried out using the Western lightning chemiluminescence reagents kit (PerkinElmer Life Sciences).

Gas Chromatography and Mass Spectrometry Analysis—Contractile proteins of soleus and EDL muscles were separated using a linear SDS-PAGE gradient gel (10–20%) and were then transferred on polyvinylidene difluoride membrane. We cut off of the membrane individually each protein band corresponding to the different contractile proteins and submitted them to a reductive β-elimination (0.1 M NaOH, 1 M BH$_3$Na) for 18 h at 45 °C. The reaction was stopped on ice by the addition of acetic acid to bring it to pH 5–6. Methyborates were eliminated by co-distillation with methanol followed by evaporation under N$_2$. Dried samples containing alditols were treated with acetic anhydride at 100 °C for 4 h. Peracetylated alditols were then extracted in chloroform.

Chloroformic phase was analyzed by gas chromatography on a 30 m × 0.32-mm BPX 70 column, with a cyanopropyl (polysilphenylene-siloxane) stationary phase. A peracetylglucosaminitol standard was analyzed using the same conditions.

For gas chromatography/mass spectrometry analysis, the gas chromatography separation was performed on a Carlo Erba GC 8000 gas chromatograph equipped with a 25 m × 0.32-mm CP-Sil5 CB low bleed/MS capillary column and 0.25-μm film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 260 °C, and the samples were analyzed using the following temperature program: 90 °C for 3 min, next 5 °C/min up to 260 °C, followed by a plateau of 20 min at 260 °C. The column was coupled to a Finnigan AutoMass II mass spectrometer. Analyses were performed in the EI mode (ionization energy 70 eV; source temperature 150 °C) and in the positive CI mode, in the presence of ammonia (ionization energy 150 eV, source temperature 100 °C). A reference standard sample of peracetylated glucosaminitol was used to secure these identifications.

For each experiment, a 5-mm fiber segment was isolated from the skinned biopsy of soleus. A silk thread was tied at each extremity, allowing the mounting of the fiber in an experimental chamber with constant stirring, initially filled with R solution. The fiber was held at one end by small fixed forceps and at the other end by a clamp connected to a strain gauge (force transducer Fort 10 (World Precision Instruments), sensitivity 10 V/g). The resting sarcomere length was measured by means of a helium/Neon laser (Spectra Physics) directed perpendicular to the long axis of the fiber. Next, the fiber was stretched to ~120% of resting length to allow maximal isometric tension development upon ionic activation. The resulting sarcomere length (2.6 ± 0.04 μm) was subsequently regularly controlled and readjusted if necessary. The output of the force transducer was amplified and recorded on a graph recorder (Gould model 6120).

**Force Measurements and Effects of GlcNAc on Calcium Properties**—All experiments were performed in a thermostatically controlled room (20 ± 1 °C). In our study, two experimental protocols were used.

In the first protocol, single fibers were bathed for 1 h in the R solution. Next, the same fibers were bathed for 1 h in the R solution containing 0.2 μM GlcNAc, 0.2 μM GalNAc, or 0.2 μM glycerol. The muscular fibers were incubated in a solution of free N-acetyl-D-glucosamine to suppress protein-protein interactions through the O-GlcNAc moiety. It is known that incubation of free GlcNAc allows a rise in protein-protein interaction depending on the O-GlcNAc moiety (39). The concentration 0.2 μM was chosen, since it is the optimal concentration to elute glycoproteins bearing O-GlcNAc by affinity chromatography on immobilized WGA, a lectin that recognizes N-acetyl-d-glucosamine.

In the second protocol, single fibers were submitted to two successive baths of 1 h each. One set of experiments corresponded to two baths in 0.2 μM glycerol, and another set corresponded to a first bath in 0.2 μM GalNAc followed by a second bath in 0.2 μM GlcNAc.

After preliminary bathings according to the two protocols described above or in the absence of bathing (control experiments), each single fiber was bathed in W solution to eliminate EGTA traces from the previously applied R solution. Next, the
fiber was activated at a level P with various pCa solutions (from 7.0 to 4.8, with a step ordinarily equal to 0.2 pCa units). Each steady state submaximal tension P was followed immediately by a maximum contraction Po ensured by pCa 4.2 solution that contained enough calcium to saturate all troponin C sites. The tensions P were expressed as a percentage of the maximal tension Po and reported as tension/pCa (T/pCa) relationships. Finally, the fiber was relaxed in R solution. All solutions contained the tested molecules (i.e. GlcNAc, GaINAc, or glycerol) except for the control experiments. For some experiments, fibers were washed, and a new control T/pCa relationship was performed to determine the reversibility of the effects observed in the presence of GlcNAc. Fibers were rejected if force declined during a sustained contraction or decreased by more than 20% during the whole experiment and if T/pCa were not completely achieved.

The T/pCa relationship provided information about the affinity of the contractile apparatus for Ca^{2+} that was represented by the pCa_{50} value (50% of maximal Ca^{2+} tension responses). Two other important parameters could be determined from the T/pCa relationship: the threshold for activation by Ca^{2+} as an indicator of the calcium sensitivity of the contractile system and the steepness of the T/pCa reflecting the cooperativity between the different regulatory proteins within the thin filament. The steepness of the T/pCa was determined by the Hill coefficients n_1 and n_2, calculated according to the following equation (40): P/Po = (([Ca^{2+}]/K)^n_1) / (1 + ([Ca^{2+}]/K)^n_2), where P/Po is the normalized tension and K is the apparent dissociation constant (pK = -log K). n_1 corresponds to P/Po > 50%, and n_2 corresponds to P/Po < 50%. Curves were fitted with the Hill parameters (n_1 for P/Po > 50%, and n_2 for P/Po < 50%) according to the relation y = (1/(1 + 10^{-n} \times (pCa_{50} - pCa))) \times 100.

Electrophoresis—All fibers were dissolved in 20 μl of SDS lysis sample buffer and stored at −20 °C until electrophoretic analysis. The samples were heated at 90 °C for 3 min just prior to the electrophoresis. A 10–20% linear gradient SDS-PAGE was used to obtain a good separation of the contractile proteins. Proteins were silver-stained.

Statistical Analysis—All of the data were reported as means ± S.E. The statistical significance of the difference between means was determined using the t test. Differences at or above the 95% confidence level were considered significant.

RESULTS
Contractile Protein Enrichment

In order to specifically study the O-GlcNAc modification of contractile proteins, we performed an enrichment of contractile proteins from a total extract of skeletal muscles before the purification of O-GlcNAc proteins by affinity chromatography. Skeletal muscle powder was homogenized in a high ionic solution allowing an extraction of contractile proteins (41). A pellet of contractile proteins was obtained by this protocol. Electrophoretic analysis of this pellet allowed easy identification of myosin heavy and light chains (essential and regulatory light chain), actin, and tropomyosin isoforms as previously reported (42–45) and shown in Fig. 1.

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| (kDa) | 1 | 2 |
|------|---|---|
| 175 | Myosin heavy Chains |
| 83  | Actin |
| 62  | Tropomyosin isoforms |
| 47.5 | MLC1 |
| 32.5 | Myosin light chains |
| 25  |
| 16.5 |
| 6.5  |

FIGURE 1. Contractile protein enrichment from gastrocnemius skeletal muscle. SDS-PAGE 10–20% silver-stained of total extract muscle (lane 1) and contractile proteins (lane 2).

O-GlcNAc Contractile Protein Enrichment

Contractile proteins obtained as described above from soleus (Fig. 2, lanes 1 and 3) and EDL (Fig. 2, lanes 4 and 6) muscles were purified by WGA-immobilized affinity chromatography to retain O-GlcNAc-modified proteins. Fractions corresponding to flow-through (lanes 1 and 4), GalNAc wash (lanes 2 and 5), and GlcNAc elution (lanes 3 and 6) were analyzed on 10–20% SDS-PAGE after silver staining (Fig. 2).

The proteins that were retained in the WGA-immobilized affinity chromatography were also present in the flow-through fractions (Fig. 2, lanes 1 and 4), indicating that only a fraction of the contractile proteins retained were modified with O-GlcNAc. The low signal obtained with GalNAc wash, which eliminates nonspecific interactions between proteins and WGA (Fig. 2, lanes 1), for soleus and EDL, respectively, indicated that proteins retained on WGA column were not eluted or were very slightly eluted with GalNAc monosaccharide. Proteins were first identified according to their migration, and then the identification was performed by MALDI-TOF spectrometry analysis and confirmed by tandem mass spectrometry analysis (Table 1). Profiles of contractile proteins purified on a WGA column were similar for soleus and EDL, indicating that the same proteins are O-GlcNAc-modified in slow and fast skeletal muscles (i.e. myosin heavy chain, actin, and the specific muscle type isoforms of MLC1 and MLC2).

Confirmation of the O-GlcNAc Nature of Contractile Proteins

We confirmed the modification of these proteins by immunoblotting with the RL2 anti-O-GlcNAc monoclonal antibody (Fig. 2B). MHC, actin, and the MLCs were recognized by the antibody. A slight detection was observed for MLC2s compared with MLC2f. However, RL2 is known to bind to some but not all O-GlcNAc proteins. In fact, this antibody recognizes only specific patterns of O-GlcNAc. Therefore, the lack of interaction or the slight interaction does not indicate that the protein is not O-glycosylated. We also performed immunoblotting using the horseradish peroxidase-WGA. The pattern of O-GlcNAc proteins was similar to that obtained with the RL2 antibody (not shown). Moreover, incubation of the RL2 antibody as well as

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![Figure 2. O-GlcNAc contractile protein purification. A, SDS-PAGE 10–20% and silver staining of contractile proteins purified on WGA affinity chromatography. The purification of O-GlcNAc-modified proteins was performed in slow soleus (lanes 1–3) and fast EDL muscles (lanes 4–6). Lanes 1 and 4, flow-through fraction; lanes 2 and 5, 0.2 M GalNAc washing; lanes 3 and 6, 0.2 M GlcNAc elution. B, the glycosylation of proteins by O-GlcNAc was checked using the anti-O-GlcNAc antibody RL2 in soleus (lane 2) and EDL (lane 1) contractile proteins as well as in the presence of 0.5 M GlcNAc for the soleus (lane 3).](image)

In 0.2 M GlcNAc, the calcium activation properties of the contractile apparatus were confirmed by mass spectrometry analysis. Fig. 3B represents the fragmentation spectrum obtained for this compound. All ions obtained after mass spectrometry analysis corresponded to fragmentation ions of peracetylgulosamininitol (Fig. 3C). Results obtained with analysis of peracetylalditols confirm that myosin heavy chains, actin, and myosin light chains (essential and regulatory) are O-GlcNAc-modified in slow soleus and fast EDL muscles.

**Contractile Experiments**

**Characterization of the Glycosylation of the Biopsies Used for Contractile Experiments**—To determine whether the skinning protocol of the muscle biopsies could affect the glycosylation of proteins, we analyzed the proteins purified by an affinity chromatography on immobilized WGA beads from skinned biopsies of 2 and 4 weeks compared with control soleus homogenate by SDS-PAGE. The electrophoretic analysis revealed no difference in the pattern of O-GlcNAc-modified proteins even in 4-week biopsies compared with the soleus homogenate as illustrated (Fig. 4A, inset).

**Effect of Hyperosmolarity on Contractile Properties**—Before analysis of the effect of incubation of a skinned fiber in 0.2 M GlcNAc, we examined the effects of hyperosmolarity on contractile properties of skinned fibers (n = 7). Single skinned fibers from soleus were bathed for 1 h in 0.2 M glycerol either once or twice as described under “Experimental Procedures.” The T/pCa relationships of the skinned fibers in the presence of 0.2 M glycerol are illustrated in Fig. 4A. The calcium activation parameters, Ca\(^{2+}\) activation threshold (pCa threshold), pCa\(_{0.5}\) and Hill coefficients n\(_1\) and n\(_2\) of these fibers are indicated in Table 2. T/pCa relationships of skinned fibers (Fig. 4A) and the different parameters (Table 2) were not modified in the presence of 0.2 M glycerol even after two exposures to glycerol (data not shown). Thus, the hyperosmolarity had no effect on the calcium activation properties of the contractile apparatus.

**Effect of GlcNAc on Calcium Activation Properties**—One hour of incubation in the R solution in control conditions or 0.2 M GlcNAc produced a decrease of Po by 9.21 ± 4.98 and 11.85 ± 3.82%, respectively. These results indicated that in a saturated Ca\(^{2+}\) concentration, the maximal tension developed by the fibers was not modified by GlcNAc.

In 0.2 M GlcNAc, the T/pCa relationship was shifted toward lower pCa values (Fig. 4B, n = 11). pCa threshold values were significantly lower in the presence of GlcNAc compared with

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**TABLE 1**

Identification of O-GlcNAc-modified proteins using MALDI-TOF mass spectrometry analysis

| Name   | Score (Protein Prospector) | Number of matched peptides | Percentage of coverage sequence |
|--------|-----------------------------|-----------------------------|-------------------------------|
| MHC    | 6.48e+4                     | 18                          | 11                            |
| MHCf   | 7.08e+6                     | 13                          | 54                            |
| MHCs   | 1.73e+3                     | 12                          | 69                            |
| Actin  | 7.59e+7                     | 12                          | 67                            |
| MLC2f  | 5.60e+4                     | 10                          | 27                            |
| MLC2s  | 41 0 2 7                   | 71 2 6 7                   | 31 2 6 9                   |
| MLC1s  | 41 8 1 1                   | 71 2 6 7                   | 31 2 6 9                   |

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In 0.2 M GlcNAc, horseradish peroxidase-WGA in the presence of 0.5 M GlcNAc inhibited the detection of O-GlcNAc proteins (Fig. 2B, lane 3).
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A

B

C

FIGURE 3. Gas chromatography and mass spectrometry analysis of peracetylated alditols released from the protein first separated by SDS-PAGE. A, gas chromatogram of peracetylated alditols released of MLC2s. One of the analyzed compounds presented a retention time of 32.13 min, corresponding to the retention time of a peracetylglicosaminitol standard. B, fragmentation spectrum of the compound presented a retention time on gas chromatography of 32.13 min. C, fragmentation of peracetylglicosaminitol and fragment mass obtained.

control conditions (from \(\rho\text{Ca} 6.36\) to 6.67, respectively), and the \(\rho\text{Ca}_{50}\) values decreased significantly (from \(\rho\text{Ca} 5.59\) to 5.76, respectively) (Table 3). These results showed that the incubation in GlcNAc decreased the calcium activation parameters of the skinned fibers. The slopes of the \(T/\rho\text{Ca}\) curves were not significantly different in control conditions and in the presence of GlcNAc. Indeed, the \(n_1\) and \(n_2\) parameters were not modified (Table 3). This indicates that 0.2 M GlcNAc had no effect on the cooperativity between the different regulatory proteins within the thin filament. This effect was reversible. Indeed, when single fibers were bathed in R solution after a single bath in 0.2 M GlcNAc, the \(T/\rho\text{Ca}\) relationship was shifted toward higher \(\rho\text{Ca}\) values, the calcium activation parameters being similar to the control values (\(n = 3\); Fig. 5B).

Effect of GalNAc on Calcium Activation Properties—To demonstrate the specificity of the effects of GlcNAc on the calcium activation properties, we studied the effects of GalNAc, an epimere of GlcNAc, at a concentration of 0.2 M. As illustrated in Fig. 4C and in Table 4, 0.2 M GalNAc had no effect on the \(\rho\text{Ca}\) threshold or on the \(\rho\text{Ca}_{50}\) (\(n = 5\)). Sucrose, which was also tested, was without effect on the calcium activation parameters of skinned fibers (data not shown).

On the contrary, the application of 0.2 M GalNAc after an application of 0.2 M GalNAc shifted the \(T/\rho\text{Ca}\) relationship toward lower \(\rho\text{Ca}\) values (Fig. 5A, \(n = 6\)). As illustrated in Table 5, this shift in the presence of GlcNAc was associated with a significant decrease in \(\rho\text{Ca}_{50}\) values (from \(\rho\text{Ca} 5.59\) to 5.87, respectively). Moreover, a slight decrease of the \(n_2\) and increase of the \(n_1\) parameters after GlcNAc exposure were measured when compared with values in 0.2 M GalNAc.

These results clearly demonstrated that the effect of GlcNAc on calcium activation properties was specifically due to the N-acetylgoucosamine.

Electrophoretic Profiles of Single Fibers from Soleus after an Incubation in the Presence of 0.2 M Glycerol, GalNAc, or GlcNAc—To determine whether the effect of GlcNAc could be due to the loss of regulatory contractile proteins, we studied the electrophoretic profile of muscle fibers bathed either in GlcNAc, GalNAc, or glycerol or in control conditions.

As illustrated in Fig. 6, the electrophoretic patterns of proteins after silver staining were similar in control fibers (lane 1) and fibers bathed in GlcNAc (lane 2), GalNAc (lane 3), or glycerol (lane 4), respectively. These data indicated that there was no detectable loss of proteins consequent to the incubation of the fibers in the presence of GlcNAc.

DISCUSSION

This is the first time that it was demonstrated that the O-GlcNAc modification could be involved in the skeletal muscle contractile mechanism, in particular by modulating contractile protein-protein interactions.

Our results demonstrated that key contractile proteins were O-GlcNAc-modified (i.e. myosin heavy and light chains and actin). The O-GlcNAc nature of these contractile proteins was confirmed by immunoblotting as well as an analysis of O-glycans released after reductive \(\beta\)-elimination by mass spectrometry coupled with gas chromatography. Since these contractile proteins were also detected in the flow-through fractions, only one part of these proteins was modified. However, it was clear that the fraction of each protein modified by O-GlcNAc was not negligible. Some of these proteins, MLC2 and MLC1, corresponded also to phosphoproteins. Indeed, 30% of MLC2 slow and fast isoforms are phosphorylated in soleus and EDL, respectively (46). The level of endogenous RLC phosphorylation was shown to be a crucial determinant of the \(\text{Ca}^{2+}\) sensitivity of force development. Moreover, increased phosphorylation of myosin light chains was associated with slow-to-fast transition in rat atrophied soleus (46). These data showed that post-translational modifications can play an important role even if a large part of the protein is not constitutively modified. Post-translational phosphorylation have been demonstrated to be...
FIGURE 4. Effect of 0.2 M glycerol, 0.2 M GlcNAc, and 0.2 M GalNAc on the calcium activation parameters of skinned fibers from soleus muscle. A. T/PcA relationships in control conditions (○, continuous curve, n = 7) and after one exposure to glycerol (○, discontinuous curve, n = 7). Inset, characterization of the glycosylation of the skinned biopsies. Shown is 10–20% SDS-PAGE and silver staining of proteins purified on lectin WGA immobilized on agarose. Lane 1, biopsy skinned since 4 weeks; lane 2, soleus homogenate. B. T/PcA relationships of soleus skinned fibers were determined before (○, continuous curve, n = 11) and after one exposure to GlcNAc 0.2 M (○, discontinuous curve, n = 11). C. T/PcA relationships of soleus fibers before (○, continuous curve, n = 5) and after one exposure to GalNAc (○, discontinuous curve, n = 5). Curves were fitted with the Hill parameters (n₁ for P/Po > 50% and n₂ for P/Po < 50%).

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involved in the differential epitope expression of the slow MHC in adult rabbit masseter muscle (47). The authors proposed a modulation by O-N-acetylglucosaminylation of the phosphorylated states of slow MHC. This hypothesis is strengthened by our demonstration that MHC was modified by O-GlcNAc. The existence of contractile proteins modified by O-GlcNAc led us to suppose that O-GlcNAc could be involved in contractile protein polymerization like actin and myosin, as has been demonstrated for polymerization of filament (30), or in the modulation of contractile protein-protein interaction, like MHC and MLCs, and therefore modulate calcium activation properties of muscle fibers. Therefore, we studied calcium activation properties of muscular fibers in the presence of GlcNAc to determine whether interactions through this post-translational modification could be involved in the contractile physiology. Indeed, since we tested the effect of GlcNAc on skinned fibers that had lost the free intracellular components, the effect of GlcNAc could not be attributed to an increased glycosylation of proteins through the activation of O-GlcNAc transferase but rather by protein-protein interaction through O-GlcNAc removing. Our results demonstrated that N-acetyl-D-glucosamine at 0.2 M, a concentration that is sufficient to elute the proteins retained on a WGA-immobilized chromatography column and therefore to suppress interactions through O-GlcNAc, led to a decrease of calcium sensitivity and affinity, whereas cooperativity of proteins of the thin filament was not modified. Neither glycerol, sucrose, nor GalNAc could reproduce this effect. This indicates that the decrease in calcium

### TABLE 2
Calcium activation parameters of skinned fibers exposed to 0.2 M glycerol (n = 7)

|            | One incubation in 0.2 M glycerol | Two incubations in 0.2 M glycerol |
|------------|----------------------------------|----------------------------------|
|            | Control | Glycerol | Control | Glycerol |
| $pC_{a_{1/2}}$ | $6.74 \pm 0.09$ | $6.61 \pm 0.12$ | $6.71 \pm 0.09$ | $6.61 \pm 0.08$ |
| $pC_{a_{50}}$  | $5.77 \pm 0.04$ | $5.73 \pm 0.05$ | $5.71 \pm 0.03$ | $5.64 \pm 0.03$ |
| $n_1$      | $2.46 \pm 0.20$ | $3.40 \pm 0.72$ | $2.62 \pm 0.12$ | $2.41 \pm 0.13$ |
| $n_2$      | $2.62 \pm 0.28$ | $2.64 \pm 0.29$ | $2.55 \pm 0.26$ | $2.88 \pm 0.24$ |

**TABLE 3**
Calcium activation parameters of skinned fibers exposed to 0.2 M GlcNAc (n = 11)

|            | Control | 0.2 M GlcNAc |
|------------|---------|-------------|
|            |         |             |
| $pC_{a_{1/2}}$ | $6.67 \pm 0.09$ | $6.36 \pm 0.10^a$ |
| $pC_{a_{50}}$  | $5.76 \pm 0.03$ | $5.59 \pm 0.03^a$ |
| $n_1$      | $2.78 \pm 0.21$ | $2.89 \pm 0.18$ |
| $n_2$      | $2.63 \pm 0.18$ | $3.27 \pm 0.27$ |

* Significant differences with control conditions, $p < 0.01$.  

### TABLE 4
Calcium activation parameters of skinned fibers exposed to 0.2 M GalNAc (n = 5)

|            | Control | 0.2 M GalNAc |
|------------|---------|-------------|
|            |         |             |
| $pC_{a_{1/2}}$ | $5.79 \pm 0.01$ | $5.80 \pm 0.02^a$ |
| $pC_{a_{50}}$  | $2.09 \pm 0.22$ | $2.85 \pm 0.13^a$ |
| $n_1$      | $3.40 \pm 0.17$ | $3.34 \pm 0.26$ |

* Significant differences with control conditions, $p < 0.05$.  

### TABLE 5
Calcium activation parameters of skinned fibers exposed to 0.2 M GalNAc and 0.2 M GlcNAc (n = 6)

|            | 0.2 M GalNAc | 0.2 M GlcNAc |
|------------|-------------|-------------|
|            |             |             |
| $pC_{a_{1/2}}$ | $6.42 \pm 0.04$ | $6.28 \pm 0.03$ |
| $pC_{a_{50}}$  | $5.87 \pm 0.04$ | $5.59 \pm 0.04^a$ |
| $n_1$      | $2.08 \pm 0.22$ | $2.84 \pm 0.13^a$ |
| $n_2$      | $4.29 \pm 0.31$ | $2.74 \pm 0.24^a$ |

* Significant differences with GalNAc incubation, $p < 0.01$.  

**FIGURE 5.** Effect of 0.2 M GlcNAc and 0.2 M GalNAc on the calcium activation parameters of skinned fibers. A, T/Pcα relationships of fibers submitted to a first exposure to GalNAc (●, continuous curve, n = 6) followed by a second exposure to GlcNAc (○, discontinuous curve, n = 6). B, T/Pcα relationships of soleus fibers before (●, continuous curve, n = 3) after one exposure to GlcNAc (○, discontinuous curve, n = 3), and after washing (dashed curve). Curves were fitted with the Hill parameters ($n_1$ for $P/P_0 > 50\%$ and $n_2$ for $P/P_0 < 50\%$).

**FIGURE 6.** Analysis of the contractile protein expression pattern of skinned fibers exposed to GlcNAc, GalNAc, and glycerol. Electrophoretic profile after SDS-PAGE and silver staining of single skinned fibers from control lanes (1) and fibers exposed to 0.2 M GalNAc (lane 2), 0.2 M GlcNAc (lane 3), or 0.2 M glycerol (lane 4).
sensitivity was not a consequence of the increase in osmolarity and was specific for GlcNAc. Since we could not reproduce the effect of GlcNAc by other sugars, we postulated that the effect was rather a consequence of protein-protein interaction through O-GlcNAc removal. However, we cannot totally exclude a specific effect of GlcNAc through an unknown mechanisms involved in the development of contraction. The decrease in calcium sensitivity consequent to the incubation of skeletal muscle fibers in a solution of N-acetyl-d-glucosamine cannot be attributed to the loss of proteins, since the electrophoretic pattern of contractile proteins is not modified in the presence of O-GlcNAc. This observation could explain why the reversibility of the effect of GlcNAc was complete; after washing the proteins, can interact again through the O-GlcNAc motif. The consequence of such a decrease of calcium sensitivity and affinity in presence of GlcNAc would be a loss of force. Our results seemed to indicate that O-GlcNAc modifications could be involved in the modulation of force in skeletal muscle through a modulation of protein-protein interactions. We could then propose that variations in the level of protein glycosylation could play a role in the modulation of force development in skeletal muscle. Decrease of O-GlcNAc level was measured in atrophied soleus (32). This decrease could be involved in the loss of force observed in atrophied muscles. Troponin C, a subunit of the troponin complex, plays a key role in the contraction, acting as a Ca$^{2+}$ sensor to switch on tension development when the intracellular Ca$^{2+}$ rises, thereby modulating the Ca$^{2+}$ activation characteristics of muscle skinned fibers, especially pCa$_{50}$ (48–50). TnC could therefore be involved in the effect of GlcNAc. However, we have not been able to determine whether this protein was O-GlcNAc. Indeed, low expression of regulatory protein troponin results in a lack of detection after classical staining following SDS-PAGE.

It has been reported that the loss of MLC2 could decrease calcium sensitivity (51). Since we have demonstrated that MLC2 as well as MHC were O-GlcNAc-modified, the decrease in calcium sensitivity observed in presence of GlcNAc might involve an inhibition of myosin heavy chain and MLC2 interactions through this moiety. Thus, it has been demonstrated that the myosin heavy chain of rabbit muscle could be phosphorylated by a cascin kinase II at its amino-terminal head after removal of all of the light chains (52). The author proposed that some of the phosphorylated sites might be already occupied by O-GlcNAc.

Some of the O-GlcNAc proteins identified in this work are also phosphophosphorys. In particular, regulatory myosin light chain or MLC2 is phosphorylated in vivo by the myosin light chain kinase or myosin light chain kinase. In skeletal muscle, phosphorylation sites of MLC2 are located on serine 14 for slow MLC2 and serine 15 for fast MLC2 (53). This phosphorylation has been demonstrated to produce an increase in the calcium sensitivity of muscular fibers (i.e. inducing a shift of the force-pCa curve toward lower calcium values) (54–57). In addition, the level of endogenous RLC phosphorylation was shown to be a crucial determinant of the Ca$^{2+}$ sensitivity of force development. Glycosylation of MLC2 could balance this phosphorylation effect and therefore decrease the Ca$^{2+}$ sensitivity of muscle fibers and contribute to the modulation of actin-myosin interaction. Moreover, MLC2 phosphorylation is also involved in a structural role of contributing to assembly of myofibril filaments. During fibrillogenesis, myosin light chain kinase phosphorylates the regulatory light chain of myosin, enabling patterned assembly of myosin thick filaments (58). We could postulate that the balance glycosylation/phosphorylation of MLC2 could be involved in the modulation of myosin assembly. Thus, the precise identification of the sites modified by O-GlcNAc could clarify how phosphorylation and glycosylation might modulate the activity of MLC2.

In conclusion, our results clearly showed that O-linked N-acetylglucosaminylataion could play a role in contractile activity of skeletal muscle. Indeed, key proteins of contraction were O-GlcNAc-modified, and free N-acetyl-d-glucosamine decreased calcium sensitivity and calcium affinity of skeletal muscle fibers presumably by inhibiting protein-protein interactions dependent on O-GlcNAc. Thus, this paper presented for the first time data indicating a potential role of O-GlcNAc in contractile activity regulation.

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