Identification of O-linked N-Acetylglucosamine Proteins in Rat Skeletal Muscle Using Two-dimensional Gel Electrophoresis and Mass Spectrometry*

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O-linked N-acetylglucosaminylolation (O-GlcNac) is a regulatory post-translational modification of nucleo-cytoplasmic proteins that has a complex interplay with phosphorylation. O-GlcNac has been described as a nutritional sensor, the level of UDP-GlcNac that serves as a donor for the uridine diphospho-N-acetylglucosamine:polypeptide β-N-acetyl-glucosaminyltransferase being regulated by the cellular fate of glucose. Because muscular contraction is both dependent on glucose metabolism and is highly regulated by phosphorylation/dephosphorylation processes, we decided to investigate the identification of O-GlcNac-modified proteins in skeletal muscle using a proteomic approach. Fourteen proteins were identified as being O-GlcNac modified. These proteins can be classified in three main classes: i) proteins implicated in the signal transduction and in the translocation between the cytoplasm and the nucleus or structural proteins, ii) proteins of the glycolytic pathway and energetic metabolism, and iii) contractile proteins (myosin heavy chain). A decrease in the O-GlcNac level was measured in the slow postural soleus muscle after 14-day hindlimb unloading, a model of functional atrophy characterized by a decrease in the force of contraction. These results strongly suggest that O-GlcNac modification may serve as an important regulation system in skeletal muscle physiology. Molecular & Cellular Proteomics 3:577–585, 2004.

There are many ways for a cell to increase the complexity of its proteome from DNA to functional proteins. Among them, post-translational modifications such as phosphorylation, glycosylation, or acetylation provide additional levels of functional complexity to the cell’s proteome. Carbohydrates share a real structural diversity and are attached to proteins through two main types of linkage, respectively asparagine (N-) or serine/threonine (O-) residues. The structural diversity of the carbohydrates allows them to ensure very specific and selective interactions with other molecules. O-linked N-acetylglucosaminyl moieties (O-GlcNac)1 constitute an abundant and dynamic reversible form of glycosylation for numerous cytoplasmic and nuclear proteins (for review, see Refs. 1–4). The monosaccharide N-acetylglucosamine is linked to serine or threonine residues of a protein by the UDP-GlcNac-peptide-β-GlcNac transferase (OGT) (5–7) using UDP-GlcNac as a sugar donor and can be removed by the N-acetyl-β-D-glucosaminidase (O-GlcNacase) (8–10). The concentration of UDP-GlcNac is highly sensitive to glucose levels and depends on the hexosamine pathway (11, 12). OGT and O-GlcNacase appear to regulate the attachment and removal of O-GlcNac and could be compared with the kinase/phosphatase system in phosphorylation process (13). Indeed, phosphorylation and O-GlcNac modification are often reciprocal at the same or at neighboring hydroxyl moieties, and O-GlcNac appears as a regulatory modification that has a complex dynamic interplay with phosphorylation. This relationship between O-GlcNac and O-phosphate, called the “Yin-Yang” process, has been demonstrated on the total level of cellular proteins (14, 15) but also on isolated proteins (16, 17). Many O-GlcNac proteins have been identified to date: they belong to various classes of proteins including cytoskeletal components (18, 19), hormone receptors (16), transcriptional factors (20–24), kinases (7), signaling molecules (25), nuclear pore proteins (26), and viral proteins (27), suggesting that O-GlcNac may be implicated in several key cellular systems (transcription, nuclear transport, and cytoskeletal structure). Numerous evidence suggests the importance of O-GlcNac in

1 The abbreviations used are: O-GlcNac, O-linked N-acetylglucosaminylolation; WGA, wheat germ agglutinin; HRP, horseradish peroxidase; HU, hindlimb unloading; EDL, extensor digitorum longus; DTT, dithiothreitol; IAA, iodoacetamide; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; OGT, UDP-GlcNac-peptide-β-GlcNac transferase; O-GlcNcase, N-acetyl-β-D-glucosaminidase; IPG, immobilized pH gradient; ECL, enhanced chemiluminescence; TLC, thin-layer chromatography; TBS, Tris-buffered saline; 2D, two dimensional; AcN, acetonitrile; TFA, trifluoroacetic acid.
many pathologies including diabetes, cancer, neurodegenerative diseases, and also in adaptation processes, underlying its crucial role in cell life (for review, see Ref. 13).

The muscular contraction phenomenon is both dependent on glucose metabolism and is highly regulated by phosphorylation/dephosphorylation processes. Moreover, mammalian skeletal muscle fibers display a great potential of adaptation that results from the ability of muscle fibers to adjust their molecular, functional, and metabolic properties in response to altered functional demands, such as changes in neuromuscular activity or mechanical loading (28). Indeed, we have previously shown slow-to-fast functional transitions induced by hindlimb unloading (HU) that encompassed slow-to-fast transitions in the isoform composition of myosin heavy chain (29) as well as in other key proteins involved in the muscle contraction (30, 31). These transitions were associated with aerobic-oxidative to glycolytic metabolic changes (32) that involved both variations in the glucose metabolism and phosphorylation/dephosphorylation events (33, 34). Moreover, HU applied for 14 days on the slow-twitch antigravitational soleus muscles was involved in both variations in the glucose metabolism and phosphorylation/dephosphorylation events (33, 34). Additionally, HU that results from the ability of muscle fibers to adjust their molecular, functional, and metabolic properties in response to altered functional demands, such as changes in neuromuscular activity or mechanical loading (28).

Starting from the hypothesis that O-GlcNAc may contribute to the biological functions of some muscular proteins, using a glycoproteomic approach we identified O-GlcNAc-modified proteins in rat gastrocnemius muscle, a typical fast muscle that is composed of both fast and slow skeletal fibers. This study demonstrated that O-GlcNAc proteins are abundant in the muscle, and we also determined the variation in the total level of O-GlcNAc proteins in a rat after HU, a model of muscle atrophy. The results suggested that O-GlcNAc could play an important biological function in muscular physiology.

**EXPERIMENTAL PROCEDURES**

**Biochemicals**—Agarose immobilized-wheat germ agglutinin (WGA), extravidin-biotin peroxidase staining kit, bovine galactosyltransferase, and all chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO); sequencing-grade modified trypsin was obtained from Promega (Madison, WI); immobilized pH gradients (IPG) of 3–10 linear, UDP-[3H]Gal, mixture of antiproteases, and enhanced chemiluminescence (ECL) Western blotting detection reagent were obtained from Amersham Pharmacia Biotech (Piscataway, NJ); ZipTip-C18 pipette tips were obtained from Millipore (Bedford, MA); PN-Gase F was obtained from New England Biolabs (Beverly, MA); Duracryl 30/08, IPG loading buffer, and IPG rehydration buffer were obtained from Genomic Solutions (Cambridgeshire, United Kingdom); Vivaspin concentrators were obtained from Vivascience (Hannover, Germany); anti-O-GlcNAc antibody (RL-2) was obtained from Affinity BioReagents (Golden, CO); nitrocellulose sheets were obtained from AdvanTec MFS (Pleasanton, CA); Maxiclean cartridges C18 were obtained from Alltech (Deerfield, IL); MicroBCA protein assay reagent kit was obtained from Pierce (Rockford, IL); and thin-layer chromatography (TLC) aluminum sheet was obtained from Merck (Darmstadt, Germany).

**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).

Gastrocnemius muscles were freshly removed from male Wistar rats (n = 3) anesthetized with an intraperitoneal injection of pentobarbital sodium (3 mg.kg⁻¹), quickly frozen, and pulverized in liquid nitrogen. This muscle was chosen for its mixed composition of fibers and its large size. Specific experiments for the myosin heavy chain identification were performed in soleus and extensor digitorum longus (EDL) muscles, which are mainly composed of slow and fast fibers, respectively (32). All samples were kept at −80 °C until analyzed.

For the analysis of the O-GlcNAc levels after HU, the rats were divided randomly into two groups. One group of rats (n = 4) was subjected to 14 days of HU using the model of Morey (37) as previously described (35). A second group (n = 4) was composed of nonsuspended control animals. The two groups of animals were age- and weight-matched. After 2 weeks, animals were sacrificed and muscles were prepared as described above. The slow soleus muscle was chosen because this postural muscle presents clear slow-to-fast transitions and atrophy after HU in contrast to the fast EDL muscle.

**Affinity Chromatography on Immobilized WGA**—O-Linked N-acetyl-d-galactosamine proteins were purified with WGA affinity chromatography as previously described (38). First, muscle powder was homogenized in binding buffer (20 mM Tris/HCl, 200 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, mixture of antiproteases, pH 7.8) by ultrasonic waves (Cell Disruptor B-30; Branson Sonic Power Company, Branson, MO). The sample was then centrifuged at 19,000 × g for 1 h at 4 °C. Skeletal muscle proteins were first N-deglycosylated with peptide-N-glycosidase F (manufacturer’s specifications), and then passed through a WGA-agarose column (4 × 1 cm) at 4 °C. The column was washed with 150 ml of binding buffer, and elution was performed with 50 ml of 0.2 M GlcNAc in the same buffer. The eluted fraction was then desalted and concentrated using centrifugation on a Vivaspin concentrator (3-kDa exclusion size filter) at 4 °C. The concentrated sample was lyophilized before two-dimensional electrophoretic analysis.

**Monodimensional Immunoblot Analysis**—As already described, myosin isoforms separation was performed using a 7.5% SDS-PAGE (39). Myosin heavy chain isoforms were identified according to previous reports (29).

For revelation using the anti-O-GlcNAc antibody, membrane was saturated using a solution of 5% nonfat dry milk in Tris-buffered saline (TBS: 15 mM Tris, 140 mM NaCl, 0.05% Tween-20, pH 8.0); the antibody (dilution 1:1000 in 5% milk-TBS) was incubated at 4 °C overnight. After 5 × 10-min washes, anti-O-GlcNAc antibodies were detected using the extravidin-biotin peroxidase staining kit.

For revelation using HRP-WGA, WGA-immobilized purified glycoproteins were desialylated directly by incubating the nitrocellulose sheet in a solution of formic acid pH 2.0 at 80 °C for 30 min. Then, 4 × 10-min washes with TBS, membrane was saturated in a solution of 3% bovine serum albumin-TBS. After 4 × 10-min washes, membrane was incubated with HRP-WGA (dilution 1:10,000 in TBS) for 1 h. Membrane was finally washed 5 × 10-min in TBS. In both cases, detection was carried out using the ECL Western blotting detection reagents and hyperfilms Biomax MR to ensure optimal protein detection.
Two-dimensional (2D) Electrophoresis—For identification by peptide mass mapping, skeletal muscle O-GlcNAc proteins were first separated on 2D-PAGE. WGA-immobilized purified proteins were resuspended on 200 μl of loading buffer and 200 μl of rehydration buffer (Amersham Pharmacia Biotech), homogenized, and shaken for 1 h at room temperature. Sample was loaded on a first-dimension strip (18 cm, pI 3–10), and rehydration occurred overnight. The gel was run for 100,000 V/h during 24 h. After running, the first-dimension gel was loaded on a 10% acrylamide gel after 20 min equilibration in buffer I (1.5 μl Tris/HCl, 6 μl urea, 2% SDS, 30% glycerol, 0.01% bromophenol blue, 35 mM dithiothreitol (DTT), pH 8.8) followed by 20-min equilibration in buffer II (1.5 μl Tris/HCl, 6 μl urea, 2% SDS, 30% glycerol, 0.01% bromophenol blue, 87 mM iodoacetamide (IAA), pH 8.8). Following SDS-electrophoresis, the gel was silver stained.

“In-gel” Digestion of Proteins—Spots were excised, gel pieces were destained by reduction with a solution of 30 mM potassium ferricyanide/100 mM sodium thiosulfate, and then the pieces were washed with water. Proteins were reduced at 56 °C for 30 min with 10 mM DTT in 0.1 M NH₄HCO₃ followed by alkylation with 55 mM iAA in 0.1 M NH₄HCO₃ for 30 min at room temperature in the dark. Gel pieces were washed with 0.1 M NH₄HCO₃ for 15 min, then were dehydrated and shrunk by CH₃CN in a vacuum centrifuge.

For the “in-gel” digestion with trypsin, gel pieces were rehydrated in the digestion buffer containing 0.1 M NH₄HCO₃, 5 μl CaCl₂, and 5 ng/μl of trypsin at 4 °C for 30–45 min. The excess supernatant was removed, and the gel pieces were covered with 10–20 μl of the 0.1 M NH₄HCO₃ buffer. The digestion was performed overnight at 37 °C.

Mass Spectrometry—After “in-gel” tryptic digestion, tryptic peptides were extracted from the gel particles. After addition of 50 μl of 25 mM NH₄HCO₃, gel pieces were shaken for 15 min. Supernatant was collected. Two successive extractions were performed with 30 μl of acetonitrile (AcN/HCOOH/water (45/10/45, v/v/v)) for 20 min with shaking. Supernatants were pooled with the first aliquot of NH₄HCO₃. The last extraction was done with AcN/HCOOH (95/5, v/v) for 20 min with shaking. The extracts were pooled together and dried in a vacuum centrifuge. Samples were desalted using Zip-TipC₁₈ pipette tips; binding and washing of peptides on the Zip-Tip column were realized in 0.1% trifluoroacetic acid (TFA) in water. Elution was performed with 5 μl of 0.1% TFA in AcN/water (60/40, v/v). Samples were dried in a vacuum centrifuge, resuspended in 0.1% TFA in water.

Protein identification was carried out using peptide mass fingerprinting on a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-STR PRO). One micro-liter of desalted and concentrated sample was directly spotted on the target plate with 1 μl of freshly prepared 2,5-dihydroxybenzoic acid matrix (10 mg/ml dissolved in CH₃OH/H₂O (70/30, v/v)). Peptide mass fingerprint spectra were registered in reflecton positive ion mode. On average, 150–200 laser shots were accumulated per spectrum. Each spectrum was internally calibrated using the monoisotopic mass of trypsin, and the sample was boiled for 3 min. Radioactive precursor was eliminated by passing the sample through a column of Dowex 50 × 8 in its acetate form. The column was washed with water, and the nonretained fraction was counted after addition of Aquasafe on a Beckman LS6000TA apparatus (Beckman Coulter, Fullerton, CA). The level of O-GlcNAc labeled with [³H]Gal was determined using the specific radioactivity of UDP-[³H]Gal and the counting efficiency. This experiment is characteristic of three assays.

Reductive β-Elimination—One hundred micrograms of purified glycoproteins were used for this experiment. Classical conditions of reductive β-elimination were used (final concentrations of 0.1 M NaOH and 1 M BH₄Na). The reaction was carried out for 18 h at 45 °C. It was stopped by passing through a Dowex 50 × 8 column in its H⁺ form on ice, and the resin was then elinated by filtration on a glass fiber. Solution containing β-eliminated products was then desalted on a C₁₈ column equilibrated in 0.1% TFA in water, and elution was performed with 0.1% TFA in AcN/water (60/40, v/v). The eluted sample was dried in a vacuum centrifuge, resuspended in 10 μl of water, and analyzed by TLC. Migration of TLC was obtained in the solvent BuOH/CH₃COOH/H₂O (40/20/30, v/v/v). Revelation was developed with sulfuric orcinol.

Statistical Analysis—Values are presented as means ± SE and number of observations. Statistical analyses were performed using Student’s t test, the acceptable level of significance being set at p < 0.05.

RESULTS

Numerous Skeletal Muscle Proteins Purified on WGA-immobilized Affinity Column Are O-GlcNAc—The O-GlcNAc modified proteins from skeletal muscle were prepared using a pulverized sample of gastrocnemius muscle passed through a WGA-immobilized column in order to retain the O-GlcNAc proteins. WGA is a plant lectin that has a double specificity against both O-GlcNAc terminal residues and sialic acid. To test the specificity of the O-GlcNAc proteins purification, mono- and two-dimensional gel electrophoresis using Western blotting techniques were performed.

As illustrated in Fig. 1A, numerous proteins were enriched on a WGA-immobilized column, especially the proteins with a molecular mass higher than 45 kDa. These purified proteins were then revealed with HRP-WGA, before (Fig. 1B) and after (Fig. 1C) chemical desialylation of the purified glycoproteins: patterns were similar without desialylation or after desialylation, suggesting that WGA recognized only proteins bearing terminal GlcNAc residues in our sample and not sialic acid. No signal was detected (Fig. 1D) when WGA was incubated in the presence of 0.2 μM GlcNAc, indicating that WGA signals were specific of O-GlcNAc residues. A similar result was obtained with the anti-O-GlcNAc antibody. As observed in Fig. 1E, the anti-O-GlcNAc antibody recognized proteins purified on a WGA-immobilized affinity column, revealed after silver staining (Fig. 1A), confirming that these proteins are O-GlcNAc modified.

Fig. 2 showed that after a reductive β-elimination of glycoproteins purified on a WGA-immobilized column, there was only N-acetylglucosaminitol as product (co-migration with a...
N-acetylglucosaminitol standard), demonstrating that only O-GlcNAc proteins and not longer O-glycans were present in the sample. These control experiments demonstrated that the proteins purified on the WGA-immobilized column were O-GlcNAc proteins and not sialylated glycoproteins.

2D Gel Electrophoresis of WGA-purified Proteins—In order to identify the O-GlcNAc modification in skeletal muscle proteins purified on a WGA-immobilized affinity column, we separated the proteins using 2D gel electrophoresis.

Comparison of the 2D electrophoresis of total gastrocnemius muscle proteins (Fig. 3A) with 2D electrophoresis of muscle proteins after purification on the WGA-immobilized affinity column (Fig. 3B) showed that O-GlcNAc post-translational modification is present on numerous proteins and that a large number of O-GlcNAc proteins are normally weakly expressed in skeletal muscle. Comparative analysis of the gels in Fig. 3, B (silver staining of O-GlcNAc proteins purified on the WGA column) and C (Western blot of O-GlcNAc proteins purified on the WGA column, separated by 2D gel electrophoresis and stained with HRP-WGA after chemical desialylation) confirmed that the proteins purified on the WGA-immobilized affinity column are exclusively O-GlcNAc proteins. We must note that some spots, corresponding to O-GlcNAc proteins that are weakly expressed or possessed few O-GlcNAc sites, revealed on Fig. 3B are not detected in Fig. 3C, presumably because of the sensitivity of O-GlcNAc protein detection using lectins (i.e. WGA).

Proteomic Analysis—The best-resolved spots were excised and submitted to proteomic analysis. Table I provides the protein identification of 14 spots (circled in Fig. 3B) by MALDI-TOF mass spectrometry. These proteins may be classified in three classes: the first one corresponds to proteins involved in the signal transduction and in the translocation between the cytoplasm and the nucleus or structural proteins, such as B-crystallin (spot 1), phosphoinositide 3-kinase regulatory subunit (spot 2), protein phosphatase 2A (spot 3), mitogen-activated protein kinase kinase kinase 8 (spot 4), homolog of yeast nuclear protein localization 4 (spot 5), and serine protease inhibitor III (spot 6). Seven proteins constitute the second class and correspond to enzymes implicated in the glycolytic pathway and in the energetic metabolism, such as muscle-specific -enolase (spot 7), muscle-specific fructose bisphosphatase aldolase (spot 8), creatine kinase M (spot 9), triose phosphate isomerase (spot 10), glyceraldehyde-3-phosphate dehydrogenase (spot 11), mitochondrial malate dehydrogenase (spot 12), and carbonic anhydrase III (spot 13). The third class corresponds to proteins involved in the contractile machinery and, in particular, one of them was identified as the myosin heavy chain (spot 14).

Some of the O-GlcNAc proteins identified in the gastrocnemius displayed different isoforms separated according to differences in their pI and might represent different phosphorylated forms of the same O-GlcNAc-protein; some of them
were identified by MALDI-TOF mass spectrometry (Table I). This is the case for β-enolase, creatine kinase M, or fructose-bisphosphatase aldolase (spots 7, 8, and 9 in Fig. 3B).

All the other excised spots corresponded to proteins that were unsuccessfully identified due to their very low expression level and very low probability scores. It should be mentioned that analysis of the two large bands observed on the gel (arrows in Fig. 3B) have not been successfully attributed to known proteins. We assumed that these bands correspond to protein mixtures unresolved by 2D gel electrophoresis.

The O-GlcNAc modification of myosin, which is particularly interesting considering its key role in muscle contraction, was confirmed with immunoblot analysis using an anti-O-GlcNAc antibody. All the myosin isoforms were unambiguously identified as O-GlcNAc proteins, as illustrated in Fig. 4, lane 2. No signal was detected in Fig. 4, lane 3, when the anti-O-GlcNAc antibody was incubated in the presence of 0.2 M GlcNAc, indicating that signals observed on Western blot were specific of O-GlcNAc moieties and not aspecific signals.

**Total O-GlcNAc Level Assay**—We measured the total level of O-GlcNAc in slow soleus and fast EDL skeletal muscles, as well as in the atrophied soleus muscle. Fig. 5 shows the relative O-GlcNAc level on muscular proteins in slow and fast skeletal muscles. There is $2.83 \pm 0.03 \times 10^{-3}$ pmol of O-GlcNAc for 100 μg of proteins in the control slow skeletal muscle soleus against $2.39 \pm 0.08 \times 10^{-3}$ pmol of O-GlcNAc for 100 μg of proteins in the control fast skeletal muscle EDL. After 14 days of HU, there was a spectacular development of...
atrophy in the slow antigravitational muscle as previously described (Ref. 35, not shown). The soleus muscle weight decrease from 0.387 ± 0.013 mg/g (mg muscle/g of animal; mean ± S.E.; n = 4) in control conditions to 0.156 ± 0.009 mg/g after HU (p < 0.05); this atrophy is associated with a decrease in the O-GlcNAc level, which became similar to that found in the EDL fast muscle (2.35 ± 0.01 × 10⁻³ pmol, p < 0.05).

**DISCUSSION**

This article reports the identification of 14 O-GlcNAc cytosolic muscular proteins by proteomic approach. This is the first report concerning a possible implication of O-GlcNAc in muscle physiology. Numerous O-GlcNAc-modified proteins were stained by silver coloration on a 2D electrophoresis performed after purification on a WGA-immobilized affinity column, suggesting the abundance of proteins concerned by this post-translational modification in skeletal muscle. However, most of these proteins were not identified due to their low expression level.

**TABLE I**

O-GlcNAc proteins identified in skeletal muscle, after purification on a WGA column, separation on 2D electrophoresis and MALDI-TOF mass spectrometry analysis

| Name                                           | Mr (Da) | pI       | Score (NCBI)     | Score (Swiss-Prot) | Number of matched peptides | Percentage of coverage sequence |
|------------------------------------------------|---------|----------|------------------|--------------------|---------------------------|---------------------------------|
| Proteins implicated in signal transduction, nuclear transport, and structural proteins |         |          |                  |                    |                           |                                 |
| αB-crystallin                                  | 19 958  | 6.8      | 1.66e+5          | 3.23e+5            | 14                        | 66                              |
| Phosphoinositide-3-kinase regulatory subunit, p85 | 85532   | 5.9      | 1.24e+5          | 9.16e+3            | 8                         | 13                              |
| Protein phosphatase 2A                         | 45555   | 5.4      | 2.01e+4          | 2.70e+4            | 16                        | 43                              |
| MAP kinase kinase kinase 8                    | 52808   | 5.7      | 1.09e+5          | 1.05e+5            | 9                         | 16                              |
| Homolog of yeast nuclear protein localization 4| 68 057  | 6.0      | 1.18e+5          | –                  | 10                        | 15                              |
| Serine protease inhibitor III                  | 45555   | 5.3      | 2.54e+6          | 8.83e+6            | 16                        | 43                              |
| Proteins of glycolytic pathway and energetic metabolism |         |          |                  |                    |                           |                                 |
| Muscle specific β-enolase                      | 46 961  | 7.6      | 9.61e+3          | 5.09e+3            | 15                        | 24                              |
| Fructose bisphosphatase aldolase, muscle specific | 39 352  | 8.3      | 3.00e+8          | 1.41e+8            | 24                        | 48                              |
| Creatine kinase, M form                        | 43 019  | 6.6      | 3.05e+9          | 1.258e+9           | 26                        | 60                              |
| Triose phosphate isomerase                     | 26 921  | 6.4      | 1.42e+9          | 5.55e+8            | 30                        | 70                              |
| Glyceraldehyde-3-phosphate dehydrogenase       | 35 836  | 8.4      | 2.78e+12         | 5.39e+11           | 21                        | 47                              |
| Malate dehydrogenase mitochondrial             | 35 656  | 8.9      | 6.23e+5          | 1.96e+5            | 11                        | 22                              |
| Carbonic anhydrase III                         | 29 401  | 6.9      | 1.14e+6          | 2.99e+6            | 13                        | 53                              |
| Contractile protein                            |         |          |                  |                    |                           |                                 |
| Myosin heavy chain                             | 103 583 | 5.3      | 2.72e+4          | –                  | 14                        | 14                              |

Fig. 4. Analysis of the O-GlcNAc modification of myosin (7.5% SDS-PAGE). The different myosin heavy chain isoforms (MHC) were detected after silver staining (lane 1) or using the anti-O-GlcNAc antibody (lane 2). The specificity of the antibody was controlled in presence of 0.2 mM GlcNAc (lane 3).

**Soleus**

**EDL**

Fig. 5. Measurement of total O-GlcNAc level in the slow skeletal muscle soleus and the fast skeletal muscle EDL. Total O-GlcNAc level assay was obtained by a radioactive labeling of O-GlcNAc moieties by bovine galactosyltransferase (see “Experimental Procedures”). The assays (n = 3) were carried out in the slow skeletal muscle soleus, in the fast skeletal muscle EDL, and in the slow soleus muscle after HU. The level of O-GlcNAc was expressed in 10⁻³ pmol of O-GlcNAc for 100 μg proteins. Values are means ± S.E. Asterisks indicate significant differences with the control.
Moreover, some of the proteins corresponding to different isoforms of the same protein were separated according to their pl. Different pl could refer to different phosphorylated forms. This observation suggests that some of the proteins might be simultaneously O-GlcNAc and O-phosphate on the same or at neighboring sites. This is the case for M-creatine kinase and fructose bisphosphatase aldolase. For the β-enolase, two different isoforms have been identified on 2D gels. These two isoforms do not correspond to different phosphorylated forms, but are due to the presence or the absence of a C-terminal lysine (40); nevertheless, these two isoforms are both O-linked N-acetylglucosaminylated.

The identified proteins were classified in three distinct classes corresponding respectively to 1) proteins implicated in the signal transduction in the translocation between the cytoplasm and the nucleus or structural proteins, 2) enzymes of the glycolytic pathway and energetic metabolism, and 3) contractile proteins.

The identified O-GlcNAc proteins described in the literature are associated with many functions including cellular regulation, transcriptional machinery, protein-protein interactions, cytoskeleton proteins, protein undergoing proteasomal degradation, phosphatases, or kinases (3, 41). Some of the identified skeletal muscle proteins fall into these different classes: the homolog of yeast nuclear protein localization corresponds to proteins involved in the nuclear translocation while other proteins concern enzymatic systems or intracellular regulation pathway (protein phosphatase 2A, mitogen-activated protein kinase kinase kinase 8, and phosphoinositide-3-kinase) or structural proteins (αB-crystallin).

An interesting observation is that the predominant class of identified proteins concerns enzymes of the glycolytic pathway. Due to the advanced role of O-GlcNAc in the control of enzyme activity, we could postulate that the O-GlcNAc residue may modulate the activity of enzymes of the glycolytic pathway and consequently might be involved in the regulation of glucose metabolism in skeletal muscle. It is noteworthy that the concentration of the donor sugar UDP-GlcNAc has been demonstrated to be highly sensitive to glucose levels (11, 12). Moreover, correlation between the level of O-GlcNAc and glycogen content has been measured in skeletal muscle (36).

A role of O-GlcNAc in a protein-protein interaction process (41) should also be considered. There is evidence for the existence of interactions between β-enolase and creatine kinase M, two key enzymes of the muscle metabolism, in the cytosol of skeletal muscle cells (42). β-Enolase is known to interact with many other proteins: indeed, the association between β-enolase and aldolase or pyruvate kinase is of high affinity, and β-enolase also binds the sarcomeric protein tropomin (40). These specific interactions between glycolytic enzyme complexes and the contractile apparatus, which could be modulated by phosphorylation and/or O-GlcNAc, could allow the formation of ATP at its site of utilization. Similarly, muscular creatine kinase, an important enzyme catalyzing the reversible transfer of a phosphate moiety between ATP and creatine, has been described in myofibrils as a structural protein of M-band (43) as well as αB-crystallin that interacted with actin and desmin intermediate filaments to increase the stability of Z-bands (44).

O-GlcNAc has also been associated with protection against degradation (45). Indeed, The O-GlcNAc site in rhesus monkey lens αB-crystallin, Thr179, which is conserved in rat lens and rat heart αB-crystallin as well as in human, bovine, mouse, and hamster αB-crystallin, could prevent the αB-crystallin from protein degradation (46). In slow soleus muscle, the disuse atrophy is associated with a marked reduction in αB-crystallin expression (47). It is possible that a decrease in O-GlcNAc level in this protein induces its degradation, resulting in a disintegration of myofibrillar proteins.

The major observation concerns the identification of heavy myosin as being O-GlcNAc modified. Myosin, a key contractile protein that constitutes the major component of the thick filament in mammalian muscle fiber, is involved in the actomyosin cross-bridge necessary for the force development in skeletal muscle. Different isoforms of myosin heavy chain encoded by different genes have been identified (48). In addition to diversity in myosin heavy chain expression based on different gene expression, a different type of diversity, called epitope diversity, has been described (49): it was suggested that this epitope diversity might be a result of post-translational modifications of the myosin heavy chain. It has also been demonstrated that the myosin heavy chain of rabbit muscle could be phosphorylated by a casein kinase II at its amino-terminal head (50). Moreover, the phosphorylation of the slow myosin heavy chain has been reported as a potential post-translational modification being involved in the epitope diversity (51). These authors proposed that some of the phosphorylated sites might be already occupied by O-linked N-acetylglucosaminylation. Our results clearly argue in favor of this hypothesis. Because an epitope was localized in the light meromyosin and could be implicated in the thick filament assembly, we might suggested that O-GlcNAc, and to a large extent the Yin-Yang process, may exist and could be involved in the regulation of the polymerization of myosin in the thick filament assembly. However, the O-GlcNAc sites still remain to be determined and a role of this post-translational modification in the regulation of the actomyosin complex and the regulation of muscle contraction must be suspected.

It is noteworthy that among the identified proteins, six of them are known to be involved in the muscle plasticity and adaptation to new physiological conditions. Thus, myosin heavy chain, muscle creatine kinase, αB-crystallin, as well as glyceraldehyde-3-phosphate dehydrogenase have been reported to be four earlier markers of muscle disuse (52, 53), while induction of carbonic anhydrase III (54) as well as effect on muscle specific enolase (55) have been demonstrated in muscle after denervation. This could indicate that the O-GlcNAc modifications are involved in the muscle plasticity and
rapid adaptation to new physiological conditions. The variation in the O-GlcNac level measured after HU argues for the implication of this post-translational modification in the muscle plasticity. Indeed, there is a clear decrease in the O-GlcNac level in the slow soleus muscle, which is particularly affected by HU. Moreover, because the decrease in normalized O-GlcNac level becomes similar to that in fast muscle, the variation of O-GlcNac might be an indicator of the phenotypical slow-to-fast transition. To conclude, further studies will be needed to grasp the implication of O-GlcNac in muscle physiology and plasticity.

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