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In vivo Phosphoproteome of Human Skeletal Muscle Revealed by Phosphopeptide Enrichment and HPLC-ESI-MS/MS

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Abstract

Protein phosphorylation plays an essential role in signal transduction pathways that regulate substrate and energy metabolism, contractile function, and muscle mass in human skeletal muscle. Abnormal phosphorylation of signaling enzymes has been identified in insulin resistant muscle using phosphoepitope-specific antibodies, but its role in other skeletal muscle disorders remains largely unknown. This may be in part due to insufficient knowledge of relevant targets. Here, we therefore present the first large-scale *in vivo* phosphoproteomic study of human skeletal muscle from 3 lean, healthy volunteers. Trypsin digestion of 3-5 mg human skeletal muscle protein was followed by phosphopeptide enrichment using SCX and TiO_2. The resulting phosphopeptides were analyzed by HPLC-ESI-MS/MS. Using this unbiased approach, we identified 306 distinct *in vivo* phosphorylation sites in 127 proteins, including 240 phosphoserines, 53 phosphothreonines and 13 phosphotyrosines in at least 2 out of 3 subjects. In addition, 61 ambiguous phosphorylation sites were identified in at least 2 out of 3 subjects. The majority of phosphoproteins detected are involved in sarcomeric function, excitation-contraction coupling (the Ca^{2+}-cycle), glycolysis and glycogen metabolism. Of particular interest, we identified multiple novel phosphorylation sites on several sarcomeric Z-disc proteins known to be involved in signaling and muscle disorders. These results provide numerous new targets for the investigation of human skeletal muscle phosphoproteins in health and disease and demonstrate feasibility of phosphoproteomics research of human skeletal muscle *in vivo*.

Introduction

Reversible phosphorylation is a key regulatory mechanism controlling the activity of enzymes in cellular signaling processes in higher organisms[1]. In skeletal muscle, phosphorylation plays a critical role in signal transduction pathways that regulate substrate and energy metabolism, excitation-contraction coupling, sarcomeric function, muscle mass and fiber type composition in response to physiological variations in mechanical stress, physical activity and circulating levels of substrates, hormones and inflammatory factors[2-4]. Several studies of phosphorylation events in human skeletal muscle have investigated the molecular mechanisms that regulate

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exercise/contraction-stimulated glucose transport, fiber type composition, and mitochondrial biogenesis[5], or underlie impaired insulin signaling to glucose transport and glycogen synthesis in patients with type 2 diabetes and high-risk individuals[6-9]. However, such studies are hypothesis-driven and limited to known phosphorylation sites on enzymes for which phosphoepitope-specific antibodies are available. Recognizing that up to one-third of all eukaryotic proteins are phosphorylated[10], it is likely that aberrant phosphorylation of muscle enzymes plays a much greater role than hitherto demonstrated in the etiology of skeletal muscle pathologies such as insulin resistance, diabetes, age- and cancer-related muscle wasting, as well as inherited myopathies and muscle dystrophies.

Recently, HPLC-ESI-MS/MS has emerged as a valuable tool to characterize phosphorylation without requiring radioactive labeling. Compared with the use of phosphoepitope-specific antibodies, this represents an unbiased approach capable of monitoring cellular phosphorylation events in the absence of *a priori* knowledge. One of the main obstacles to phosphoproteome studies is, however, the low abundance of the phosphopeptides relative to the high abundance non-phosphopeptides in a complex mixture. Thus, among approximately 1000 proteins identified in recent proteomic studies of human skeletal muscle[11-12], only 35 phosphorylation sites in 24 proteins were detected. For this reason enrichment of phosphopeptides prior to MS analysis has been a major focus in phosphoproteome studies. Available approaches for phosphopeptide enrichment include strong cation exchange chromatography (SCX)[13-14], immobilized metal affinity chromatography (IMAC)[15], metal oxide chromatography using titanium dioxide (TiO_2)[16-18], calcium phosphate precipitation[19], zirconia[20] and alumina[21], as well as immunoprecipitation with anti-phosphotyrosine or anti-phosphoserine/threonine antibodies[22]. Most large-scale phosphoproteome studies reported so far were carried out in either cell culture or animal models. Moreover, greater than 10 mg of lysate proteins was frequently used as starting materials, which is impractical in most human studies, where the amount of tissue that can be obtained from sequential biopsies is limited. To date, human *in vivo* studies have examined the phosphoproteome for platelets[23], liver[24], T lymphocytes[25], and brain[19]. Nonetheless, no large scale *in vivo* human skeletal muscle phosphoproteome study has yet been reported.

Here we report the first global analysis of the *in vivo* phosphoproteome of human skeletal muscle from 3 lean, healthy volunteers, using 3-5 mg of muscle protein from each. Skeletal muscle lysate was subjected to in-solution trypsin digestion, followed by phosphopeptide enrichment using SCX and TiO_2 and analysis of the resulting peptides by HPLC-ESI-MS/MS. This approach resulted in the identification of 306 distinct phosphorylation sites in 127 proteins in at least 2 out of 3 subjects. In addition, 61 ambiguous phosphorylation sites were identified in at least 2 out of 3 subjects. To our knowledge, these results represent the largest catalog of the human skeletal muscle phosphoproteome to date, providing novel targets for the investigation of human skeletal muscle phosphoproteins in health and disease.

Experimental Procedures

Subjects

The skeletal muscle sample used for the proteomics analyses in this study was obtained from 3 lean healthy male volunteers (age: 32-48 years; body weight: 82.6-85.4 kg; body height: 1.82-1.91 m, BMI: 23.4-24.9 kg/m^2) with normal glucose tolerance and no family history of type 2 diabetes. The purpose, nature and potential risks of the study were explained to the participant, and written consent was obtained before participation. The protocol was approved by the Institutional Review Boards of Arizona State University or the University of Texas Health Science Center at San Antonio.

Protein isolation

A percutaneous needle biopsy of the *vastus lateralis* muscle was obtained under local anesthesia, and the muscle biopsy specimen (~30-50 mg) was immediately blotted free of blood, frozen, and stored in liquid nitrogen until use. The muscle biopsy was homogenized while still frozen in an ice-cold buffer (10 μl/mg tissue) consisting of (final concentrations): 20 mM HEPES, pH 7.6; 1mM EDTA; 250 mM sucrose, 2 mM Na_3VO_4; 10 mM NaF; 1 mM sodium pyrophosphate; 1 mM ammonium molybdate; 250 μM PMSF; 10 μg/ml leupeptin; and 10 μg/ml aprotinin. After homogenized by a polytron homogenizer on maximum speed for 30 sec, the homogenate was cooled on ice for 20 min and then centrifuged at 10,000 × g for 20 min at 4 °C; the resulting supernatant containing 2 mg of lysate supernatant proteins (Solution 1) was used for in-solution digestion. The resulting pellet was dissolved by adding 400 μl 6 M guanidine HCl, centrifuged, and 1 mg of the resulting proteins (Solution 2) was used for in-solution digestion. Protein Solution 1 and Solution 2 were processed in parallel during the following steps. Protein concentrations were determined by the method of Lowry[26].

In-solution trypsin digestion

Solid urea was added into the protein Solution 1 and Solution 2, respectively to a final concentration of 8 M. Proteins were reduced in 10 mM (final concentration) dithiothereitol (DTT), shaken 1 hr at 600 rpm at 55 °C, cooled down to room temperature, and alkylated in 50 mM (final concentration) freshly made idoacetamide (IDA) at room temperature for 45 min in the dark. The resulting mixture was diluted 8 fold in 40 mM ammonium bicarbonate so that the final concentration of urea and guanidine HCl was lower than 1 M. Proteomics grade Trypsin (Sigma Chemical Co., St. Louis, MO) in 40 mM ammonium bicarbonate was added at a substrate: trypsin ratio of 1:100. The digestion was allowed to proceed at 37 °C overnight and was terminated by the addition of 5% formic acid (FA) to adjust the pH value below 4.0. Appropriate amounts of 5% HFBA and 100% ACN were added to achieve final concentration of 0.05% HFBA and 2% ACN. The resulting peptide mixtures were desalted by solid-phase extraction (Sep-Pak C18 1cc cartridge, Waters corporation, Milford, MA) after sample loading in 0.05% heptafluorobutyric acid:2% ACN (v/v) and elution with 400 μl 50 % ACN:1% FA (v/v) and 400 μl 80%ACN:1%FA (v/v), respectively. The two eluates were combined and the sample volume was reduced to approximately 100 μl by vacuum centrifugation.

Enrichment of phosphopeptides

200 μl of 0.05% heptafluorobutyric acid (HFBA)/1% FA/2%ACN were added into the desalted peptide sample and the resulting solution was first separated on a strong cation exchange column (SCX) and then followed by TiO_2 enrichment. Briefly, the resulting solution containing both nonphosphorylated and phosphorylated peptides was loaded onto a 1ml HiTrap SP HP SCX column (GE Sciences, Torrance, CA). Flow through was collected and 8 salt steps were applied to elute peptides off the column. The salt steps contained 1ml each of the following concentration of ammonium formate solution in 0.05% HFBA/2%ACN: 12, 21, 35, 58, 95, 170, 330 and 1000 mM. 3 mg TiO_2 beads (GL sciences, Japan) were placed in a 1.5 ml tube, washed twice by 0.05% HFBA/80%ACN and 300 mg lactic acid was added into the tubes. Nine such tubes were prepared and the resulting 9 fractions from the SCX column were added into each of the 9 tubes. The 9 mixtures were shaken at room temperature for 30 min and washed with 50 μl 300 mg/ml lactic acid in 0.05%HFBA/80%ACN and followed by 100 μl 0.05%HFBA/80%ACN and 100 μl 0.05%HFBA, respectively. Phosphopeptides were eluted off the TiO_2 beads using 0.5% ammonia and 5% ammonia, respectively. Each eluate was acidified using 50% formic acid to pH =3 and 2 μl 5%HFBA containing three standard peptides, Angiotension 1, Angiotension 2 and Influenza Hemagglutinin (HA) Peptide (250 fmol/μl, Sigma Chemical Co., St. Louis, MO), was added.

Mass spectrometry

HPLC-ESI-MS/MS was performed on a hybrid linear ion trap (LTQ)-Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer (LTQ FT; Thermo Fisher, San Jose, CA) fitted with a PicoView™ nanospray source (New Objective, Woburn, MA). On-line capillary HPLC was performed using a Michrom BioResources Paradigm MS4 micro HPLC (Auburn, CA) with a PicoFrit™ column (New Objective; 75 μm i.d., packed with ProteoPep™ II C18 material, 300 Å). Samples were desalted using an on-line Nanotrap (Michrom BioResources, Auburn, CA) before being loaded onto the PicoFrit™ column. HPLC separations were accomplished with a linear gradient of 2 to 27% ACN in 0.1% FA in 70 min, a hold of 5 min at 27% ACN, followed by a step to 50% ACN, hold 5 min and then a step to 80%, hold 5 min; flow rate, 300 nl/min. A “top-10” data-dependent tandem mass spectrometry approach was utilized to identify peptides in which a full scan spectrum (survey scan) was acquired followed by collision-induced dissociation (CID) mass spectra of the 10 most abundant ions in the survey scan. The survey scan was acquired using the FTICR mass analyzer in order to obtain high resolution and high mass accuracy data.

Data analysis and bioinformatics

Tandem mass spectra were extracted from Xcalibur “RAW” files and charge states were assigned using the Extract_MSN script (Thermo Fisher, San Jose, CA). The fragment mass spectra were then searched against the IPI_HUMAN_v3.59 database (80,128 entries, http://www.ebi.ac.uk/IPI/) using Mascot (Matrix Science, London, UK; version 2.2). The false discovery rate was determined by selecting the option to search the decoy randomized database. The search parameters used were: 10 ppm mass tolerance for precursor ion masses and 0.5 Da for production masses; digestion with trypsin; a maximum of two missed tryptic cleavages; fixed modification of carboamidomethylation; variable modifications of oxidation of methionine and phosphorylation of serine, threonine and tyrosine. Probability assessment of peptide assignments and protein identifications were made through use of Scaffold (version Scaffold_2_00_06, Proteome Software Inc., Portland, OR). Only peptides with ≥ 95% probability were considered. Proteins that contained identical peptides and could not be differentiated based on MS/MS analysis alone were grouped. Multiple isoforms of a protein were reported only if they were differentiated by at least one unique peptide with ≥ 95% probability, based on Scaffold analysis.

Only phosphorylation sites detected in 2 out of 3 subjects were considered as a potential phosphorylation site in human skeletal muscle and were subjected to further verification by manual inspection of corresponding MS/MS spectra. Criteria used for manual validation include: 1. assignment of the majority of the high intensity peaks; 2. for a phosphopeptide with multiple Ser/Thr/Tyr residues, detection of at least two unique fragment ions with a signal to noise ratio grater than 5 for a specific phosphorylation isoform.

Gene Ontology annotation of human proteins was downloaded from Gene Ontology Annotation (GOA) Databases (http://www.ebi.ac.uk/GOA, version 55.0). This GOA human database contains 33,731 distinct proteins and 172661 GO associations. In addition, GO hierarchy information (version 52) was downloaded from www.geneontology.com. Human GO associations and GO hierarchy information were assembled into a new database by an in-house script written using MATLAB. IPI IDs, gene names, UniProt and SwissProt IDs of identified proteins were input into the database to obtain GO associations and GO hierarchy information. Furthermore, gene IDs for identified phosphoproteins were manually inputted into www.genecards.org and www.ncbi.nlm.nih.gov/IEB/Research/Acembly to retrieve additional subcellular localization information.

Results

Human Skeletal Muscle Phosphoproteome

We combined strong cation exchange (SCX) with titanium dioxide (TiO_2) and enriched phosphopeptides from 3-5 mg of human muscle lysate from 3 lean healthy subjects (Figure 1). HPLC-ESI-MS/MS analysis revealed 498, 498, 475 non-redundant phosphorylation sites (with ≥95% confidence as assessed by Mascot and Scaffold analysis) from Subject 1-3, respectively. Total number of non-redundant phosphorylation sites from the three subjects is 879. In order to improve the confidence of the sites identified, we required that a phosphorylation site be detected in at least 2 out of 3 subjects. There were 412 such phosphorylation sites. Manual inspection of the MS/MS spectra corresponding to these 412 phosphorylation sites for verification revealed that 306 sites could be uniquely assigned, while 61 were ambiguous, mainly due to the fact that there are several potential phosphorylation sites in one phosphopeptide.

The 306 unique *in vivo* phosphorylation sites were localized in 127 proteins/protein groups and included 240 phosphoserines, 53 phosphothreonines, and 13 phosphotyrosines, giving a phosphoserine/phosphothreonine/phosphotyrosine ratio of ~18:4:1. (Table 1). The 61 ambiguous phosphorylation sites were localized in 51 proteins. Out of these 51 proteins, 17 had only ambiguous sites (Table 1). Therefore, in total, we have identified 367 phosphorylation sites in 144 phosphoproteins/phosphoprotein groups from at least 2 out of 3 subjects, where a phosphoprotein group consists of phosphoproteins that share the exact same identified phosphopeptide. In addition, 265 proteins without phosphorylation sites were identified in at least 2 out of 3 subjects. As a result, 35% of all proteins identified were phosphoproteins using phosphopeptide enrichment with SCX and TiO_2, which compares to about 2% in our previous proteomics studies of human skeletal muscle without phosphopeptide enrichment[11,12]. The false discovery rate, as assessed by Mascot searching of a randomized database, was 2% at the peptide level. A detailed list of all proteins identified in this study together with their IPI ID, sequence coverage, and number of unique peptides assigned to each protein are provided in Supplemental Table 1. In addition, we have included the following in Supplemental Table 2 for each peptide identified (with ≥95% confidence as assessed by Mascot and Scaffold analysis): modifications, flank residues, precursor mass, charge and mass error observed, and the best Mascot score.

Among the proteins identified in this study, a number of entries derived from the protein identification searches had multiple IPI IDs. In many cases, assignment of multiple IDs results from the potential presence of protein isoforms that could not be distinguished on the basis of unique peptides. Proteins with multiple IDs were therefore assigned to a “protein group”. Proteins that were assigned a unique IPI ID are listed as a single-entry protein group. This analysis resulted in 409 protein groups from at least 2 out of 3 subjects as shown in Supplemental Table 1. For protein groups with multiple IPI IDs, the mean number of amino acids in each protein sequence is listed in Supplemental Table 1.

Due to missed cleavage by trypsin, identical phosphorylation sites may appear in different peptides. Each identical phosphorylation site that appeared in different peptides was therefore grouped into one phosphorylation site group, resulting in 306 such groups that were distinctly assigned and 61 that were ambiguous. Proteins/protein groups that shared the exact same identified phosphopeptide were grouped into a phosphoprotein group, which resulted in 144 such groups (Table 1). If there is more than one IPI ID in a phosphoprotein group, due to differences in the amino acid sequence, the phosphorylation site location may be different for each protein belonging to the same phosphoprotein group. Therefore, in Supplemental Table 3, we have listed all 303 unique IPI ID for these 144 phosphoprotein groups with their respective

phosphorylation sites, providing the location of these phosphorylation sites within different isoforms of a protein.

Table 1 lists the identified isoforms of proteins defined as the 'canonical' sequence by UniProt/Swiss-prot. Out of the identified 306 distinct phosphorylation sites, only 122 have been reported in the four large phosphorylation site databases www.phospho.elm.eu.org, www.uniprot.org, www.phosphosite.org, as well as www.phosida.com (Table 1). Tandem mass spectra for three sites that were not reported in the 4 databases were included as Supplemental Figure 1.

Gene Ontology annotation and functional classification

Gene Ontology annotation (GO) and literature search revealed that 82 phosphoproteins were assigned to the cytoplasm, 67 to the nucleus, 36 to the membrane, 40 to the cytoskeleton, and 31 to the mitochondrion (Figure 2 and Supplemental Table 4). Notably, some proteins can be assigned to multiple GO terms.

Closer examination of the protein functions revealed that 38 (26%) of the phosphoproteins were sarcomeric proteins constituting different components of the contractile apparatus such as thin actin and thick myosin containing filaments, and M-line and Z-disk associated proteins (Table 1). 43 phosphorylation sites were identified on the three known giant muscle proteins, 33 in titin, 5 in obscurin and 5 in nebulin. Moreover, eleven phosphoproteins are important components of the Ca^{2+} signaling apparatus (Ca^{2+} cycle) mediating excitation-contraction coupling in skeletal muscle. Thus, 161 (53%) of the distinct phosphorylation sites in 49 (39%) phosphoproteins are directly related to the contractile function of human skeletal muscle. Another major group of phosphoproteins included 11 enzymes regulating glycolysis and glycogen metabolism. A total of 31 phosphorylation sites were identified in 7 out of the 11 major enzymes in the glycolytic pathway (Table 1). In addition, 14 phosphorylation sites were identified in 4 major enzymes of glycogen metabolism, and 14 phosphorylation sites were found on seven kinase subunits and two phosphatases subunits known to regulate the phosphorylation of glycogen synthase and phosphorylase. We also identified 15 phosphorylation sites on three members of the phosphocreatine (PCr) shuttle important for PCr-resynthesis after exercise. Several proteins belonging to the Ras superfamily, chaperones or involved in transcriptional regulation, protein biosynthesis, proteasomal degradation were also shown to be phosphorylated in skeletal muscle. Interestingly, we identified several phosphorylation sites in a subset of proteins, which have previously been shown to be overexpressed in relation to type 2 diabetes or related traits (RRAD[27], PEA15[28], OSBPL11[29], AHNAK[30]) or involved in insulin-mediated GLUT4 translocation (TRIP10[31], WNK1[32]).

Potential kinases for identified sites

Using NetworKin[33], we have created a list of predicted potential kinases for each of the 306 distinct phosphorylation sites (Supplemental Table 5). The number of distinct phosphorylation sites by predicted kinase family for all phosphoproteins and for the fraction of sarcomeric phosphoproteins is shown in Figure 3. The distribution shows a major role for the CKII, CDK5, PKA, p38MAPK, PKC and GSK3 kinase families in the identified phosphoproteins. As an example, the predicted potential kinases responsible for the phosphorylation sites identified in phosphoproteins involved in glucose metabolic processes are listed in Table 2.

Fractionation of phosphopeptides by SCX

Table 3 shows the identified unphosphorylated peptide/proteins and phosphopeptides/proteins in each SCX fraction followed by TiO_2 enrichment and HPLC-ESI-MS/MS. As described in the Experimental Section, after homogenization and before trypsin digestion, we separated the

lysate muscle proteins into two fractions: Supernatant and Pellet. For the Supernatant, the majority of the phosphopeptides were identified from the fractions #1-5 (containing 12, 21, 35, 58, 95 ammonium formate, respectively), while for the Pellet, most phosphopeptides were identified from the fractions #1-6 (containing 12, 21, 35, 58, 95, 170 ammonium formate, respectively). As expected, in the pellet fraction, the majority of the MS/MS spectra were assigned to contractile and structural muscle proteins, such as actin, myosin 1/2/7 and titin.

Discussion

Using 3-5 mg of protein of human skeletal muscle lysate for in-solution trypsin digestion combined with phosphopeptide-enrichment and high-accuracy nanospray tandem mass spectrometry, we identified 306 unique phosphorylation sites in 127 proteins from at least 2 out of 3 subjects. Of these, less than 40% (122 sites) have previously been reported in four large protein phosphorylation site databases (www.phospho.elm.eu.org, www.uniprot.org, www.phosphosite.org, as well as www.phosida.com). In addition, we identified 61 phosphorylation sites in 51 proteins that were ambiguous, of which17 proteins had ambiguous sites only. Therefore, in total, we have identified 367 phosphorylation sites in 144 proteins in human skeletal muscle.

By means of strong cation exchange (SCX) in combination with titanium dioxide (TiO_2) for phosphopeptide enrichments, 35% of the identified proteins were phosphorylated in the present study. This compares to about 2% in our previous proteomics studies of human skeletal muscle without phosphopeptide enrichment[11], indicating that our approach of the present study was indeed well-suited. Furthermore, although the absolute number of phosphorylation sites identified is low when compared to large-scale phosphoproteomic studies of human cell lines [14,18,34,35], the number of phosphorylation sites identified in the present study compares well with that of previous human *in vivo* studies that examined the phosphoproteome of platelets (564 phosphorylation sites in 270 proteins)[23], liver (274 phosphorylation sites in 168 proteins) [24], T lymphocytes (281 phosphorylation sites in 204 proteins)[25], and brain (466 phosphorylation sites in 185 proteins)[19]. Nevertheless, considering that about one-third of all proteins are phosphorylated at least at some point in time[10], further improvement of phosphopeptide-enrichment procedures and subsequent identification by high mass accuracy MS/MS are clearly warranted to provide a more comprehensive picture of the skeletal muscle phosphoproteome. In addition, as opposed to the resting conditions of the present study, stimulation of skeletal muscle by insulin or exercise may be required to increase the identification of phosphorylated signalling enzymes.

Mitochondrial proteins have been shown to account for approximately 20% of the human skeletal muscle proteome[11,12] and emerging evidence indicates that a number of these mitochondrial proteins are phosphorylated[36-39]. Furthermore, many cytosolic kinases are translocated into mitochondria in response to different stimuli[36,37]. In the present study, we identified 31 phosphoproteins that could be localized to the mitochondrion, accounting for 22% of all identified phosphoproteins. Although most of these proteins are also known to be localized to other compartments in skeletal muscle, these data suggest that mitochondrial phosphoproteins are about proportionately represented.

In the present study, we found a phosphoserine/phosphothreonine/phosphotyrosine ratio of ~18:4:1 in human skeletal muscle. Interestingly, this distribution shows a 2-5 fold higher share of phosphothreonines and phosphotyrosines compared to other human tissues *in vivo*, e.g. 90:9:1 in human liver tissue[24], and with cultured human cells, e.g. 86:12:2 in HeLa cells[35], suggesting tissue specific differences in protein phosphorylation. Clearly, this needs to be confirmed in future studies by direct side-by-side comparison of the phosphoproteome of different tissues.

The sarcomere is the basic functional unit in striated muscle contraction. Using our phosphoproteomic approach, we identified 132 distinct phosphorylation sites covering almost all components of the skeletal muscle sarcomere. This included 59 distinct phosphorylation sites on muscle- and fiber-type specific isoforms of thick filament proteins (myosins, myosin light chains, myosin regulatory light chains, myosin light chain kinase 2, and myosin-binding protein C) and thin filament proteins (α-actin, troponins, and tropomyosins), as well as 47 distinct phosphorylation sites on the three known giant muscle proteins, titin, nebulin and obscurin, and the M-band-specific proteins, myomesin-1 and M-protein. Moreover, we detected 26 distinct phosphorylation sites on several Z-disc proteins including α-actinins, γ-filamin, CapZ protein, CapZ-interacting protein, myozenin-1, myopalladin, myopodin, myotillin, telethonin and PDZ/LIM proteins. A number of studies have shown a role for phosphorylation of cardiac isoforms of titin and thin and thick-filament proteins in the modulation of sarcomeric function in heart muscle[40-41]. Information about phosphorylation of sarcomeric proteins in adult skeletal muscle is scarce, but includes phosphorylation of myomesin, titin, and myosin regulatory light chain[42-44], the latter with a positive effect on muscle contraction in fast-twitch type IIb fibers. NetworKIN analyses predicted a potential role for different kinases for the majority of the identified phosphorylation sites in sarcomeric proteins and showed possible involvement of the CDK5, GSK3, p38MAPK, CKII, PKC, and PKA kinase families. However, further studies are warranted to address the potential role of these phosphorylations in modulating the actomyosin interaction and myosin ATPase activity and the kinases involved *in vivo*.

The giant muscle proteins together with M-band and Z-disc proteins are important regulators of the assembly, organization and function of the contractile apparatus, and there is emerging evidence that many Z-disc proteins participate in important signaling pathways in both cardiac and skeletal muscle[4]. Of particular interest, we identified 2 novel phosphorylation sites, Ser15/Ser16 and Thr167, on myozenin-1, which is also known as calsarcin-2 due to its property as a calcineurin-binding protein. Very recent data provide evidence that calsarcin-2 inhibits calcineurin activity, and that calsarcin-2 deficiency increases exercise performance by activation of calcineurin and subsequent muscle fiber-type switch toward a slow-twitch and oxidative phenotype[45]. Our data demonstrate that known Z-disc interaction partners of calsarcin-2 such as α-actinin-2 and α-actinin-3, LIM domain-binding 3 (ZASP), telethonin/T-cap, γ-filamin and myotilin[4] are posttranslationally modified by phosphorylation. These findings implicate a potential role of reversible phosphorylation of these Z-disc proteins in the regulation of muscle fiber type composition, exercise performance, and muscle energy metabolism. Mutations in genes encoding sarcomeric proteins, in particular Z-disc proteins, are increasingly being recognized as causes of inherited cardiomyopathies and muscular dystrophies[4, 46]. Based on our findings, it is likely that such muscle disorders are characterized by abnormal sarcomeric protein phosphorylation, and that unravelling of the role of these phosphorylation sites in normal and diseased muscle may lead to the identification of potential targets for treatment of muscle malfunction and myopathic pain.

All muscle fibers use Ca^{2+} as their main regulatory and signaling molecule. Here we report several phosphorylation sites on the main proteins in the Ca^{2+}-signaling apparatus (the Ca^{2+}-cycle)[47]. Phosphorylation of the α-1S and β-subunits of the dihydropyridine receptor (L-type Ca^{2+}-channnel) may affect its interaction with the ryanodine receptor 1 (RYR1) (Ca^{2+}-release channel), and thus the electromechanical coupling that triggers release of Ca^{2+} from sarcoplasmic reticulum (SR) and subsequent muscle contraction[47]. PKA-mediated phosphorylation of RYR1 at the site identified in this study (Ser2843) is known to activate the Ca^{2+}-release channel. After intense exercise, this may cause “leaky” channels and impaired exercise capacity[48]. Phosphorylation of juntophilins (JPH1 and JPH2) may regulate their role in formation and function of skeletal muscle triadic junctions and their interaction with RYR1, and hence Ca^{2+}-release and contraction[49]. The Ca^{2+} pump (SERCA) is responsible for Ca^{2+}-

reuptake into SR from the cytoplasm at the expense of ATP hydrolysis, a critical step in muscle relaxation[47]. The slow-twitch skeletal muscle isoform SERCA2a is inhibited by phospholamban. However, this inhibitory effect is abolished by phosphorylation of phospholamban at the two sites identified in this study; at Ser16 by PKA and at Thr17 mediated by CamKII [47]. Phosphorylation of SERCA2a at Ser663 may contribute to the regulation of Ca^{2+} pumps in human skeletal muscle. Recent data suggest that the SR histidine-rich Ca^{2+}-binding protein (HRC) may play a key role in the regulation of SR Ca^{2+}-cycling through its direct interactions with SERCA2 and triadin, mediating a fine cross-talk between SR Ca uptake and release via RYR1 in the heart [50]. Whether HRC is located in the lumen of SR or anchored to SR membrane on the cytoplasmic side has been debated [51]. Supporting the notion that HRC, is a SR luminal protein NetworKIN predicted the luminal-located CK2 as a kinase for 4 of 6 phosphorylation sites. Aberrant phosphorylation of these main proteins in the Ca^{2+} cycle may interfere with the critical role that Ca^{2+} play for muscle function, plasticity and disease. However, further studies are needed to elucidate the functional significance of these phosphorylations sites under physiological and pathophysiological conditions.

Another important finding of the present study was that the majority of glycolytic enzymes are phosphorylated at multiple sites in human skeletal muscle *in vivo*. Most previous reports have demonstrated tyrosine phosphorylation of glycolytic enzymes (gene names: PFKM, ENO, PKM2, PGAM2, LDHA and GAPDH) either *in vitro* or *in vivo* in response to activation of tyrosine-specific kinases by growth factors (EGF, insulin) or by transformation of normal cells into cancer cells [52-56]. It has been proposed that tyrosine phosphorylation of glycolytic enzymes could play a role in the switch from oxidative phosphorylation to aerobic glycolysis (Warburg effect) that is important for cancer cell and tumor growth [56]. Our data indicate that under basal conditions, serine/threonine phosphorylation is more frequent than tyrosine phosphorylation of glycolytic enzymes in human skeletal muscle. Only a few earlier studies of mammalian liver and muscle have provided some evidence for serine/threonine phosphorylation of glycolytic enzymes (PKM2, PFKM), and suggested a role for PKA and CaMK in the regulation of these enzymes[57-58]. More recently, GAPDH was shown to be a Ca^{2+}-dependent substrate of phosphorylase kinase providing a direct link between glycogenolysis and glycolysis in skeletal muscle[59]. Ganon et al also identified 6 phospholabelled glycolytic enzymes (PKM2, ENO3, ALDOA, LDHA, PGM2, and TPI1) in rat skeletal muscle, and demonstrated an age-dependent change in the phospholabelling of the LDHA and ENO3 [38]. Our findings are supported by recent phosphoproteomic studies of human cell lines that have documented serine/threonine phosphorylation of several glycolytic enzymes, and in many cases at the same sites as those identified in the present study[14,18,19,34]. Using NetworKIN, we found that PKC were the most frequent predicted kinase family for the identified phosphorylation sites. Taken together these data support the hypothesis that glycolytic enzymes are regulated by other means than substrate fluxes. Further studies are needed to establish role of phosphorylation of glycolytic enzymes in normal and diseased human skeletal muscle.

Conclusions

Elucidating the role of protein phosphorylation in normal skeletal muscle and its abnormalities in skeletal muscle disorders has been limited due to insufficient knowledge of protein phosphorylation sites. Furthermore, identification of the phosphoproteome of human skeletal muscle *in vivo* has been challenging due to technical limitations. Using a combination of phosphopeptide enrichment techniques with HPLC-ESI-MS/MS, the present study provides the first large-scale phosphoproteome study of human skeletal muscle *in vivo* using a small clinically available tissue sample, a muscle biopsy at approximately 30-50 mg wet weight. Our results provide multiple novel protein phosphorylation sites for the investigation of human skeletal muscle in conditions of health and disease, and demonstrate feasibility for human

skeletal muscle phosphoproteome research *in vivo*. These data hold promise for future phosphoprotein research in human skeletal muscle in clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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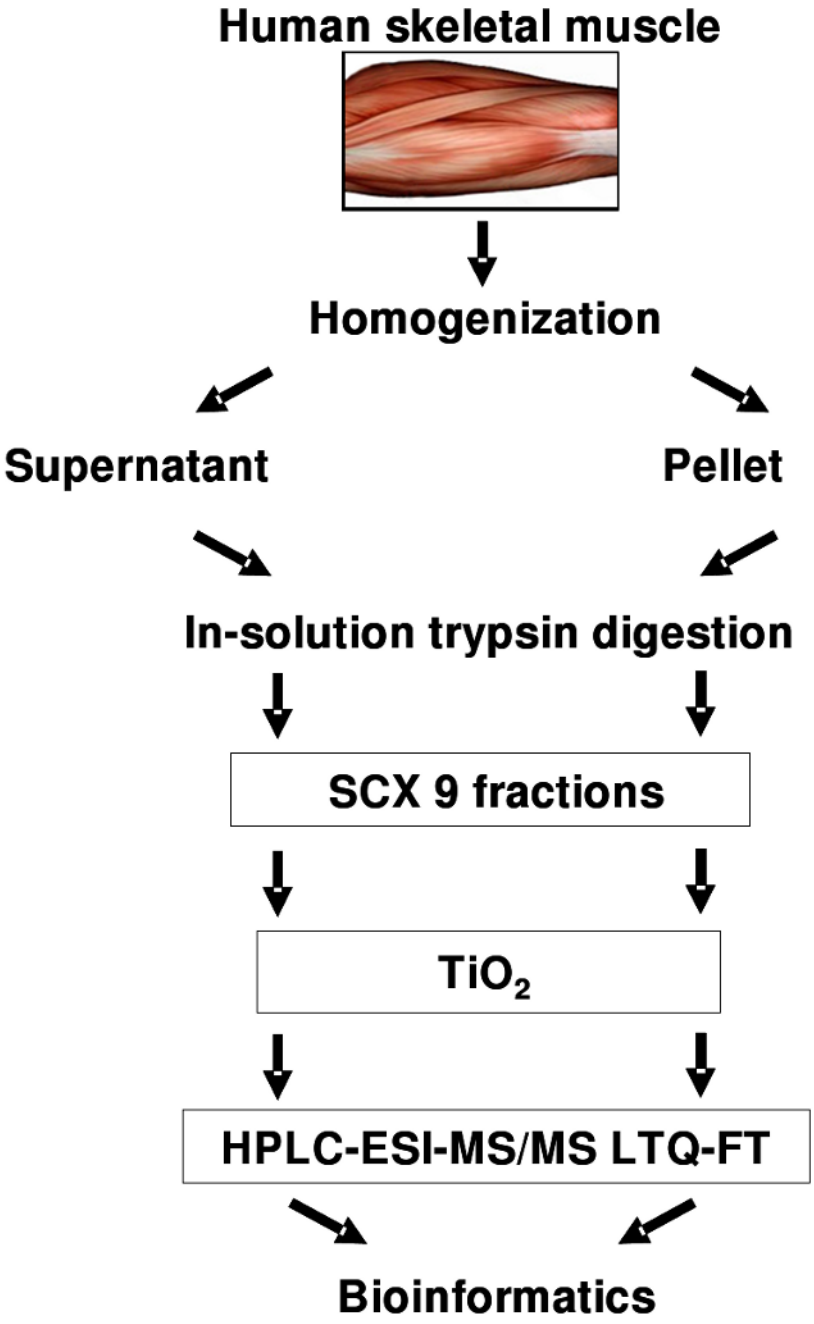

Figure 1.
Experimental workflow for the analysis of the *in vivo* phosphoproteome of human skeletal muscle.

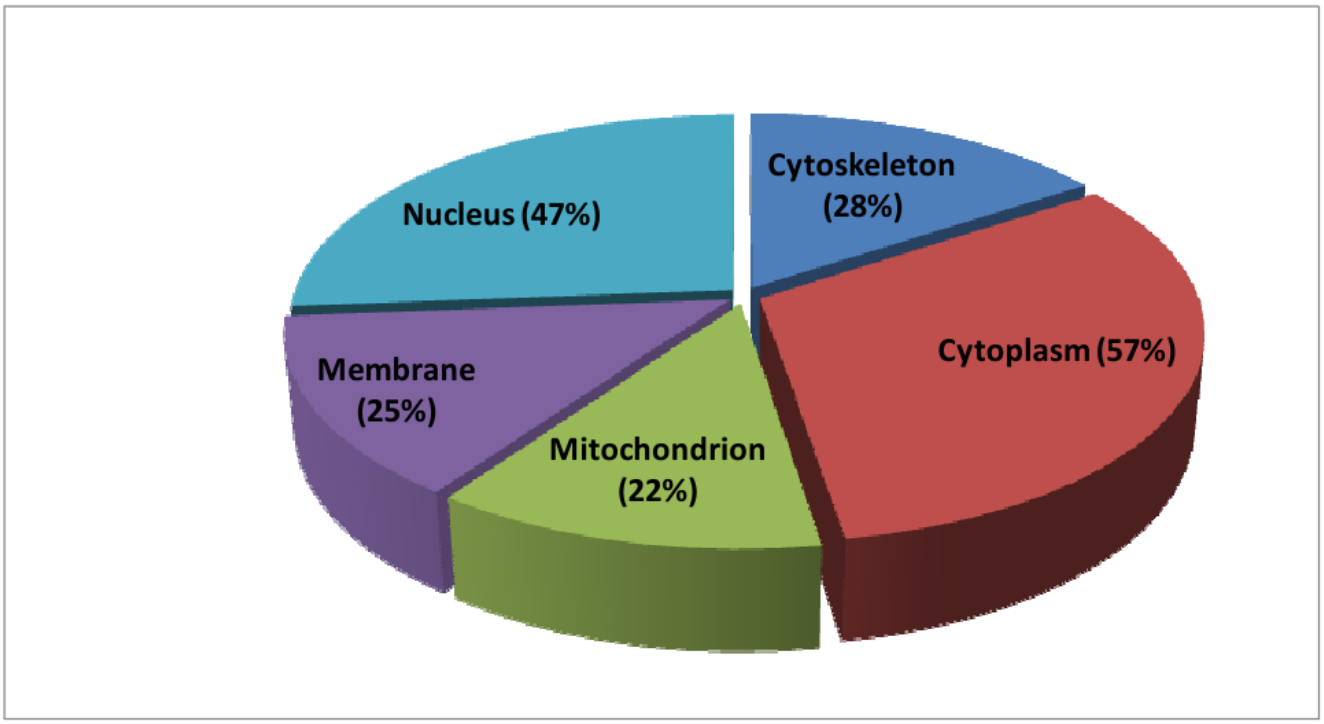

Figure 2.
Subcellular location of identified phosphoproteins in human skeletal muscle based on Gene Ontology annotation.

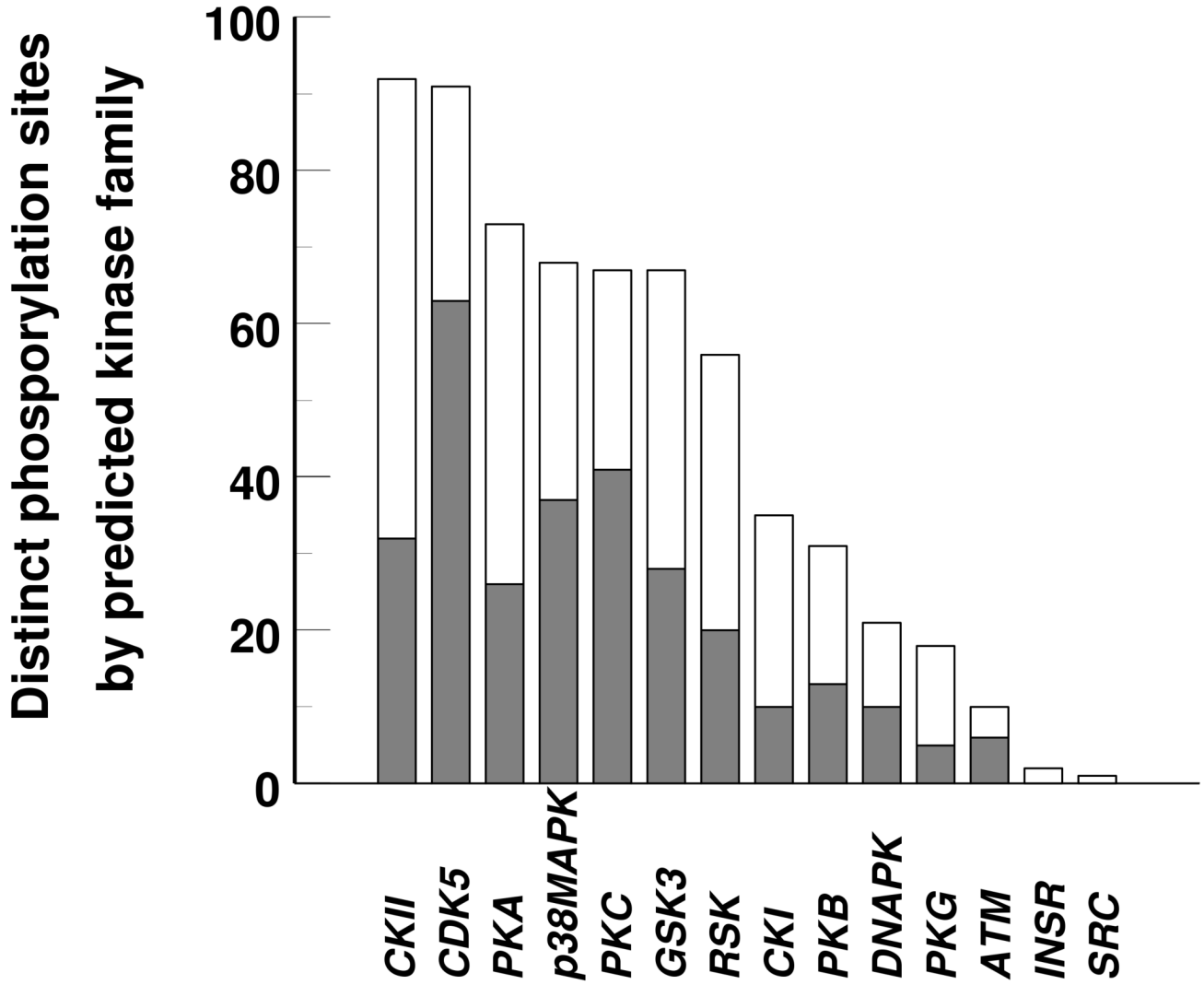

Figure 3.
Number of distinct phosphorylation sites by predicted kinase family for all phosphoproteins (white + grey bars) and for the sarcomeric phosphoproteins (grey bars).

Table 1

Phosphorylation sites identified *in vivo* in human muscle biopsy using SCX and TiO_2 followed by HPLC-ESI-MS/MS. Ambiguous sites were indicated by "/".

Gene Name	Protein Name	Phosphorylation Sites
Sarcomeric function: thin filament proteins		
ACTA1	Actin, alpha, skeletal muscle	S54*, S62, S241*/Y242*, T251, T320*, S325*/T326*,
TNNI1	Troponin I, slow skeletal muscle	S58, S183
TNNT3	Troponin T, fast skeletal muscle	S159*, S167*
TPM1	Tropomyosin alpha-1 chain	S174*, Y261*, T282*/S283*
TPM2	Tropomyosin beta chain	T79, T252*, T282/S283*
TPM4	Tropomyosin alpha-4 chain	T72
Sarcomeric function: thick filament proteins		
MYH1	Myosin-1	S1237
MYH2	Myosin-2	T51, S625, T1188, T1194, T1197, S1205*, S1239, T1243, S1263, S1267, S1305, S1308, S1368, S1482*, T1485, S1576, S1605/T1606, S1834
MYH4	Myosin-4	S732, S742, T992, S1041, S1132, S1144, Y1379*, S1480*, S1482, T1725/S1726, S1919
MYH7	Myosin-7	S738, S1102, T1188, S1275, S1299, T1309, S1510, S1600, S1718, T1721/S1722, S1894
MYL1	Myosin light chain 1, skeletal muscle	S99
MYLPF[#]	Myosin regulatory light chain 2, skeletal muscle	S15*/S16*/S17
MYL2	Myosin regulatory light chain 2, ventricular/cardiac muscle	S15*, Y118
MYLK2	Myosin light chain kinase 2, skeletal/cardiac muscle	S143, S586/S587/S588
MYBPC1	Myosin binding protein C, slow type (TrEMBL: A8KAB1)	S40, S59
MYBPC2	Myosin-binding protein C, fast-type	S44*, T46
Sarcomeric function: M-band and giant muscle proteins		
MYOM1	Myomesin 1	S65
MYOM2	Myomesin-2 (M-protein)	S597, S1057
NEB	Nebulin	T6503, S6507, S6591/S6592/S6593, S6606, S6609
OBSCN	Obscurin	S4076, T4803*/S4804/S4805, S5563, S6829, S6831
TTN	Titin	S263*, S265*, T267*, S270, T300, S302, S306, S308, S813*, S2074*, S2093, S7485, S8426, T10316, S10321*, S12009*, S12022*, S13042, S18513, Y18998*, T32998, S33000, S33013, S33071*, S33245, S33247, S33589, S33595, S33602*, S33608, S33624, T33633, S33976
TTN	Titin (Fragment)	S700
TTN	Cellular titin isoform PEVK variant 2 (Fragment)	T242
TTN	Titin isoform novex-3	S3473
Sarcomeric function: Z-disc proteins		
DES	Desmin	S28, S32*
DMD	Dystrophin	S3623*
ACTN2	Alpha-actinin-2	T237*, S431, T435, S594, S596, S840
ANK1	Ankyrin-1	S1686*
CAPZB	F-actin-capping protein beta (CapZ beta)	S289
RCSD1	CapZ-interacting protein (CAPZIP)	S216*
MYPN	Myopalladin	S643*/S644*, S813*, S818*, S928*
MYOT	Myotilin	S231, S233*
SYNPO2	Synaptopodin-2 (myopodin)	S604*, T610/S611, T626, S902*, S906*
MYOZ1	Myozenin-1 (calsarcin-2)	S15/S16, S167
FLNC	Gamma filamin (filamin C)	S2233*
CSRP3	Cysteine and glycine-rich protein 3 (MLP)	S95
PDLIM3[#]	PDZ and LIM domain protein 3 (ALP)	S148/T149/S151/S152
LDB3	LIM domain-binding protein 3, isoform 6 (ZASP)	S112, S196
Ca^{2+}-signaling		
AHNAK	Neuroblast differentiation-associated protein AHNAK	S210*/S212*, S216*, S5752*, S5762*
ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	S663*
CACNA1S	Voltage-dependent L-type calcium channel subunit a-1S	S1575
CACNB1[#]	Voltage-dependent L-type calcium channel subunit beta-1	S190/S191/S192/S193
CAMK2B	Calcium/calmodulin-dependent protein kinase IIB	S423, T463
CANX	cDNA FLJ55574, highly similar to Calnexin	S618
HRC	Sarcoplasmic reticulum histidine-rich calcium-binding protein	S119*, S157/S159*, S170*/S171, S431*, S563, S567
JPH1	Junctophilin-1	S216*
JPH2	Junctophilin-2	S484*, S486*, T490*
PLN	Cardiac phospholamban	S16*, T17*
RYR1	Ryanodine receptor 1	S2843*
Glycolysis		

Gene Name	Protein Name	Phosphorylation Sites
ALDOA	Fructose-bisphosphate aldolase A	S36*/T37, S39*, S46*, S100, S272
ENO3	Beta-enolase	S40/T41, S83, S176/S177, S282
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	T153, T182*, T184*, S210/T211*, S333
GPI	Glucose-6-phosphate isomerase	T109*, S247/T248, T250/T251
PGAM2	Phosphoglycerate mutase 2	Y92*, T96
PKM2	Pyruvate kinase isozymes M1/M2	T41, S57, S77, T80, S127, S222
TPI1	Triosephosphate isomerase;	S21*, S106, S195, S198, S204, S212
Glycogen metabolism		
GYS1	Glycogen synthase, muscle	S641*, S645*, S647*, S710*, T729*/T730/S731*
PGM1	Phosphoglucomutase-1	T115*, S117*, S134*, T507*, S509
PYGM	Glycogen phosphorylase, muscle form	Y473*, S514, Y732*
UGP2#	UTP--glucose-1-phosphate uridylyltransferase	S102/S103
Kinase/phosphatase subunits regulating GYS1 and/or PYGM		
MAPK14	Mitogen-activated protein kinase p38 alpha	T180*
PHKA1	Phosphorylase b kinase regulatory subunit alpha, skeletal muscle	S972*, S1018*/S1020*
PHKA2	Phosphorylase b kinase regulatory subunit alpha, liver	S7
PHKB	Phosphorylase b kinase regulatory subunit beta	S27*
PPP1R1A	Protein phosphatase 1 regulatory subunit 1A	S67*
PPP1R3A	Protein phosphatase 1 regulatory subunit 3A	S46*
PRKAB2#	5′-AMP-activated protein kinase subunit beta-2	S39*/S40, S182/S183*/S184*
PRKAR1A	Protein kinase A type 1-alpha regulatory subunit	S77*, S83*
PRKAR2A	Protein kinase A type 2-alpha regulatory subunit	S78*, S80*, S99*
Phosphocreatine shuttle		
AK1	Adenylate kinase 1	S38, S178, S181
CKM	Creatine kinase M-type	Y125*, S128*/S129, S164, T166, Y174*, T313, T322, S332, S372,
CKMT2	Creatine kinase, sarcomeric mitochondrial precursor	S319, S343, Y368
Actin-binding or – associated		
ANKRD2	Ankyrin repeat domain-containing protein 2	S99
CMYA5	Cardiomyopathy-associated protein 5	S3294
NEXN	Nexilin	S80*
PLEC1	Plectin-1	T4030*, S4384*/S4385*/S4386*, S4389*/S4390*, S4392*/Y4393*, S4616*
SVIL#	Supervillin	S547/S549
SYNM	Synemin	S1049*
SYNPO	Synaptopodin	S263*, S833*,
SYNPO2L	Synaptopodin 2-like protein	S264, S366, S369, S891/T892
TNS1	Tensin-1	S1177*
XIRP1	Cardiomyopathy-associated protein 1	S205, S295
Transcriptional regulators		
HDGF	Hepatoma-derived growth factor	S165*
IQWD1#	Nuclear receptor interaction protein	T654*/S657*
LMCD1	LIM and cysteine-rich domains protein 1	S16
RALY	RNA-binding protein Raly	S135*
RBMX	Heterogeneous nuclear ribonucleoprotein G	S208*
Protein Biosynthesis		
ABCF1#	ATP-binding cassette sub-family F member 1	T108*/T109*
EEF1D	Eukaryotic translation elongation factor 1 delta	S528*
EIF4G1#	Eukaryotic translation initiation factor 4 gamma 1	S990*/S992*
EIF5B	Eukaryotic translation initiation factor 5B	S214*
RPLP2	60S acidic ribosomal protein P2	S102*, S105*
Proteasomal degradation		
CUL4A	Cullin-4A	S10*
HUWE1#	E3 ubiquitin-protein ligase HUWE1	T1905*/S1907*
PSMA3	Proteasome subunit alpha type-3	S250*
Ras superfamily		
G3BP1#	Ras GTPase-activating protein-binding protein 1	S230*/S231*/S232*
RABEP1#	Rab GTPase-binding effector protein 1	S407*/T408*, S410*/T413*/S414*
RPH3A	Rabphilin-3A	T51
RRAD	GTP-binding protein RAD	S39
Chaperones		
BAG3	BAG family molecular chaperone regulator 3	S173*
HSPB1	Heat shock protein beta-1	S82*
HSPB6	Heat shock protein beta-6	S31
HSP90AB1	Heat shock protein HSP 90-beta	S255*
Related to diabetes/glucose transport		
OSBPL11	Oxysterol-binding protein-related protein 11	S172*/S174*, S184

Gene Name	Protein Name	Phosphorylation Sites
PEA15	Astrocytic phosphoprotein PEA-15	S116*
TRIP10	Cdc42-interacting protein 4	S296*, S482*/S484*
WNK1#	Serine/threonine-protein kinase WNK1	S2027*/S2029*/S2032*
Miscellaneous		
AHSG	Alpha-2-HS-glycoprotein	S204
BIN1	Myc box-dependent-interacting protein 1	S282*, S287*, T291
CA3	Carbonic anhydrase 3	S48, S50, T129, S242
CLIP1	CLIP1 protein	S193*/S195*, S200*, S204*, S348*/T350*/T351
COBL	COBL protein	S321, S324
DOCK10	DOCK10	S1596
FXYD1#	Phospholemman	S82*/S83*
GOT1#	Aspartate aminotransferase, cytoplasmic	T403/S404
KPNA3	Importin subunit alpha-3	S60*
LOC285556#	hypothetical protein	S1218/S1219/S1220
LOC389333	hypothetical protein	S260, S267/S268, T776/S777, S855
LMNA	Lamin-A/C	S390*, S392*/T394*/S395*
LSM14A	Protein LSM14 homolog A	S216*
MAP4	Microtubule-associated protein 4	S280*, T521*, S1073*
MB	Myoglobin	T68, T71, S145
MDH1	Malate dehydrogenase (TrEMBL: B7Z3I7)	S129, Y137, S206, S259
MPST	MPST protein	S15, S17
NAGK	N-acetyl-D-glucosamine kinase	S76*
NDRG2	Protein NDRG2	T330*, S332*, S335*, S338*, S346*/T348*/S350*
NME1/2	Nucleoside diphosphate kinase A/B	T94
NSFL1C	NSFL1 cofactor p47	S114*
NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinases substrate	S19*
OPTN	Optineurin	S342*
PCYTA1	Choline-phosphate cytidylyltransferase A	S362*
PEBP1	Phosphatidylethanolamine-binding protein 1	S52*, S54*, S98/S99
PDHA1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form	T269/S270, S331*
PGRMC1#	Membrane-associated progesterone receptor component 1	Y180*/S181*
PLCL2	Inactive phospholipase C-like protein 2	T584*
SEPT2	Septin-2	S218*
SMTNL2	Smoothelin-like 2	S134, S267/S269, T274, S278, S344*
SPEG	Striated muscle preferentially expressed protein kinase	S1172*, S2014, S2015, S2109/S2110, S2128/S2129/S2130, S2410, S2458,
VAPB#	Vesicle-associated membrane protein-associated protein B/C	S158/S159*
VDAC	Voltage-dependent anion-selective channel protein 1	T19, S240/S241
YWHAE	14-3-3 protein epsilon	Y131
YWHAG	14-3-3 protein gamma	Y133
YRDC	YrdC domain-containing protein	S21
	cDNA FLJ 58494	S412

phosphoproteins containing only ambiguous phosphorylations sites;

* known phosphorylation sites.

Table 2

Identified phosphoproteins involved in glucose metabolic processes with predicted potential kinases responsible for the sites.

Swissprot/Tremble ID	Site	Kinase Family	Motif Score	Context Score	Substrate Gene Name	Kinase Gene Symbol	Kinase description	Surrounding amino acid sequences of phosphorylation sites
P04075	S39	PKC	0.67	0.96	ALDOA	PKN1	Protein kinase N1	AADESTGsIAKRLQS
P04075	S46	RSK	0.52	0.96	ALDOA	RPS6KA1	Ribosomal protein S6 kinase alpha 1	SIAKRLQsIGTENTE
P04075	S272	CKI	0.58	0.96	ALDOA	CSNK1E	Casein kinase I, epsilon isoform	VTGITFLsGGQSEEE
P13929	T41*	CKII	0.53	0.95	ENO3	CSNK2A2	Casein kinase II, alpha' chain	AVPSGAStGIYEALE
P13929	S177	PKC	0.71	0.963	ENO3	PRKCD	Protein kinase C, delta type	ILPVGASsFKEAMRI
P04406	T184	ATM	0.52	0.95	GAPDH	ATM	Serine-protein kinase ATM	TVHAITAtQKTVDGP
P04406	T184	DNAPK	0.54	0.95	GAPDH	PRKDC	DNA-dependent protein kinase catalytic subunit	TVHAITAtQKTVDGP
P04406	S210*	PKC	0.85	0.95	GAPDH	PRKCD	Protein kinase C, delta type	LQNIIPAsTGAAKAV
P04406	T211*	PKC	0.58	0.95	GAPDH	PRKCD	Protein kinase C, delta type	QNIIPAStGAAKAVG
B7Z3I7	S129	ATM	0.54	0.96	MDH1	ATM	Serine-protein kinase ATM	ANVKIFKsQGAALDK
B7Z3I7	S129	DNAPK	0.52	0.96	MDH1	PRKDC	DNA-dependent protein kinase catalytic subunit	ANVKIFKsQGAALDK
B7Z3I7	S259	RSK	0.52	0.96	MDH1	RPS6KB1	Ribosomal protein S6 kinase 1	VIKARKLsSAMSAAK
P06744	T109	PKB	0.54	0.98	GPI	AKT1	RAC-alpha serine/threonine-protein kinase	ALRNRSNtPILVDGK
P06744	T248	PKC	0.62	0.97	GPI	PKN1	Protein kinase N1	KHFVALStNTTKVKE
P06744	T250	PKC	0.87	0.97	GPI	PRKCD	Protein kinase C, delta type	FVALSTNtTKVKEFG
P06744	T251	PKC	0.52	0.97	GPI	PKN1	Protein kinase N1	VALSTNTtKVKEFGI
P36871-1	T115	PKC	0.52	0.95	PGM1	PKN1	Protein kinase N1	AIGGIILtASHNPGG
P14618-2	S127	PKA	0.62	0.96	PKM2	PRKACB	cAMP-dependent protein kinase, beta-catalytic subunit	RTGLIKGsGTAEVEL
P14618-2	S127	CKII	0.52	0.96	PKM2	CSNK2A2	Casein kinase II, alpha' chain	RTGLIKGsGTAEVEL
P14618-2	S222	CKII	0.51	0.96	PKM2	CK2A1	Casein kinase II, alpha chain	AVDLPAVsEKDIQDL
P60174-1	S21	PKA	0.901	0.949	TPI1	PRKACB	cAMP-dependent protein kinase, beta-catalytic subunit	KMNGRKQsLGELIGT
P60174-1	S21	PKG	0.547	0.941	TPI1	PRKG1	cGMP-dependent protein kinase 1, beta isozyme	KMNGRKQsLGELIGT
P60174-1	S106	CKII	0.536	0.947	TPI1	CSNK2A2	Casein kinase II, alpha' chain	RRHVFGEsDELIGQK
P60174-1	S204	PKC	0.531	0.956	TPI1	PRKCD	Protein kinase C, delta type	VSDAVAQsTRIIYGG
P60174-1	S212	PKC	0.701	0.956	TPI1	PKN1	Protein kinase N1	TRIIYGGsVTGATCK

* ambiguous sites

Table 3

SCX fractionation of phosphopeptides.

SCX fractions	Total Peptides	Phospho Peptides	Unphosphorylated Peptides	Total Proteins	Phospho Proteins	Unphosphorylated Proteins
Supernatant flow through	96	71	25	66	58	8
1	563	109	454	197	80	117
2	734	170	564	274	108	166
3	765	201	564	249	126	123
4	856	191	665	247	107	140
5	674	119	555	214	72	142
6	419	64	355	168	44	124
7	231	42	189	121	36	85
8	91	24	67	49	21	28
Pellet flow through	598	70	528	277	51	226
1	676	164	512	289	106	183
2	673	132	541	270	86	184
3	1085	183	902	352	107	245
4	1093	186	907	333	115	218
5	713	138	575	269	90	179
6	793	115	678	245	72	173
7	557	69	488	165	48	117
8	407	16	391	110	15	95