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Novel Tyrosine Phosphorylation Sites in Rat Skeletal Muscle Revealed by Phosphopeptide Enrichment and HPLC-ESI-MS/MS

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Abstract

Tyrosine phosphorylation plays a fundamental role in many cellular processes including differentiation, growth and insulin signaling. In insulin resistant muscle, aberrant tyrosine phosphorylation of several proteins has been detected. However, due to the low abundance of tyrosine phosphorylation (<1% of total protein phosphorylation), only a few tyrosine phosphorylation sites have been identified in mammalian skeletal muscle to date. Here, we used immunoprecipitation of phosphotyrosine peptides prior to HPLC-ESI-MS/MS analysis to improve the discovery of tyrosine phosphorylation in relatively small skeletal muscle biopsies from rats. This resulted in the identification of 87 distinctly localized tyrosine phosphorylation sites in 46 muscle proteins. Among them, 31 appear to be novel. The tyrosine phosphorylated proteins included major enzymes in the glycolytic pathway and glycogen metabolism, sarcomeric proteins, and proteins involved in Ca²⁺ homeostasis and phosphocreatine resynthesis. Among proteins regulated by insulin, we found tyrosine phosphorylation sites in glycogen synthase, and two of its inhibitors, GSK-3 α and DYRK1A. Moreover, tyrosine phosphorylation sites were identified in several MAP kinases and a protein tyrosine phosphatase, SHPTP2. These results provide the largest catalogue of mammalian skeletal muscle tyrosine phosphorylation sites to date and provide novel targets for the investigation of human skeletal muscle phosphoproteins in various disease states.

Keywords

Tyrosine phosphorylation; Rat muscle; Tyrosine immunoprecipitation; HPLC-ESI-MS/MS

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1. Introduction

Protein tyrosine phosphorylation plays a key regulatory role in numerous cellular processes such as growth, proliferation, metabolic homeostasis, differentiation and aging [1]. Most tyrosine kinases are tightly negatively regulated, and only activated under specific conditions. This in part explains why tyrosine phosphorylation accounts for less than 1% of the phosphoproteome [1]. Interestingly, the identification of aberrant expression and activity of tyrosine phosphorylated proteins in multiple forms of cancer has led to the development of specific tyrosine kinase inhibitors for cancer therapy [2, 3]. This emphasizes the importance of investigating tyrosine phosphorylated proteins.

Skeletal muscle has been widely studied to understand the molecular basis for the physiological adaptation to acute exercise, endurance training and sarcopenia, as well as the pathophysiological changes underlying cancer-related muscle wasting, acquired and inherited myopathies, and insulin resistance [4-10]. In skeletal muscle, tyrosine phosphorylation of specific enzymes modulates insulin and growth factor signaling to glucose transport, glycogen synthesis, protein synthesis, and transcriptional activation [7, 9, 11, 12]. Moreover, the use of phosphopeptide-specific antibodies has demonstrated aberrant phosphotyrosine-mediated signaling in skeletal muscle of insulin resistant individuals [7, 13]. However, recent studies of the tyrosine phosphoproteome in different cell types and tissues have revealed hundreds of tyrosine phosphorylation sites, not only in the previously identified signaling proteins, but also in cytoskeletal proteins, proteins involved in RNA metabolism, and glycolytic enzymes [14-18]. This suggests that the prevalence of tyrosine phosphorylated proteins in skeletal muscle is currently underestimated, and that tyrosine phosphorylation could be involved in the regulation of various muscle processes, such as excitation-contraction coupling, sarcomeric function, muscle mass, fiber-type composition and mitochondrial function. Consequently, there is a need for appropriate mass spectrometry-based global protein characterization techniques to generate a more comprehensive picture of the role of tyrosine phosphorylation in muscle.

Using strong cation exchange and titanium dioxide for phosphopeptide enrichment followed by HPLC-ESI-MS/MS, we previously identified 306 phosphorylation sites in human skeletal muscle. However, only 13 tyrosine phosphorylation (pTyr) sites in 9 proteins were detected [19], probably due to the high abundance of cytoskeletal and contractile proteins in skeletal muscle that made it difficult to detect the low abundant tyrosine phosphorylated proteins by common phosphoproteomics [20, 21]. The combination of phosphotyrosine antibody immunoprecipitation with MS/MS-based detection has emerged as a valuable tool to comprehensively study tyrosine phosphorylation [22, 23]. At first, this has been done using antibodies to immunoprecipitate tyrosine phosphorylated proteins from cultured cells [22, 24] but recent reports indicate that immunoprecipitation of phosphotyrosine peptides, rather than proteins, improves the study of site-specific tyrosine phosphorylation [16, 17, 23, 25-30].

To our knowledge, no large scale study of *in vivo* tyrosine phosphorylation of mammalian skeletal muscle has yet been reported. In the present study, we therefore analyzed the *in vivo* tyrosine phosphorylation of rat skeletal muscle by in-solution trypsin digestion of skeletal muscle lysates, followed by phosphopeptide enrichment using immunoprecipitation of phosphotyrosine peptides and analysis of the resulting peptides by HPLC-ESI-MS/MS. This approach resulted in the identification of 87 distinctly localized tyrosine phosphorylation sites in 46 muscle proteins. To our knowledge, these results represent the largest catalog of skeletal muscle tyrosine phosphorylation sites in mammals to date, and provide novel targets for the investigation of skeletal muscle phosphoproteins in response to exercise, sarcopenia, and pathophysiological conditions such as myopathies, cachexia, and insulin resistance.

2. Material and methods

2.1. Animals

The 3 skeletal muscle samples used for the proteomics analyses in this study were obtained from 3 male Sprague-Dawley rats (Charles River, Wilmington, MA) on a regular chow diet (70% carbohydrate, 10% fat, and 20% protein of calories, Research Diets #D12450B, New Brunswick, NJ) with water ad libitum. Rats were 4 weeks of age, weighed 260-428g, and were housed one per cage in a temperature-controlled environment maintained at 21 °C with an artificial 12:12-h light-dark cycle. The animals were anesthetized with a single intraperitoneal injection of pentobarbital sodium (40 mg/kg), and the vastus lateralis muscle biopsy specimen was immediately blotted free of blood, frozen, and stored in liquid nitrogen until use. The protocol was approved by the Institutional Animal Care and Use Committee of Arizona State University.

2.2. Protein isolation

The muscle biopsies (~100 mg) were 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 Na₃VO₄; 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 5 mg of lysate supernatant proteins (Solution 1) was used for in-solution digestion. The resulting pellet was dissolved by adding 400 µl of 6 M guanidine hydrochloride, centrifuged, and 5 mg of the resulting proteins (Solution 2) were 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.

2.3. 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) dithiothreitol (DTT), shaken 1 hr at 600 rpm at 55 °C, cooled down to room temperature, and alkylated in 50 mM (final concentration) freshly made iodoacetamide (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 hydrochloride was lower than 1 M. Proteomics grade Trypsin (Sigma Chemical Co., St. Louis, MO) in 40 mM ammonium bicarbonate was added at a trypsin:substrate ratio of 1:200. 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% heptafluorobutyric acid (HFBA) and 100% acetonitrile (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% HFBA:2% ACN (v/v) and elution with 400 µl of 50 % ACN:1% FA (v/v) and 400 µl of 80%ACN:1%FA (v/v), respectively. The two eluates were combined and the sample volume was reduced to approximately 10 µl by vacuum centrifugation.

2.4. Enrichment of tyrosine phosphopeptides

Immunoprecipitation of phosphotyrosine peptides (pTyr-IP) was performed using the PhosphoScan® Kit (P-Tyr-100, #7900, purchased from Cell Signaling, Danvers, MA). Briefly, the peptide mixture from the in-solution digestion was mixed with 1.4 ml immunoprecipitation buffer (50mM MOPS, pH 7.2; 10mM sodium phosphate; 50mM NaCl). 40 µl of pTyr-100 antibody beads in 80 µl slurry were added to the resulting solution

and the mixture was incubated overnight on a rotator at 4°C. The beads were washed with the immunoprecipitation buffer three times, followed by HPLC-grade water twice. Phosphopeptides were eluted off the beads with 50 µl of 0.15% trifluoroacetic acid (TFA) twice. The two eluates were pooled. The resulting peptide mixtures were purified by solid-phase extraction (C18 OMIX C18, Agilent Technologies, Santa Clara, CA). The samples were dried by vacuum centrifugation and dissolved in 4 µl of 0.1% TFA:2% ACN (v/v), followed by HPLC-ESI-MS/MS analysis.

2.5. Mass spectrometry

The entire peptide mixture from in-solution digestion was desalted, subjected to pTyr-IP, and the resulting enriched tyrosine phosphopeptides were desalted as described above, followed by HPLC-ESI-MS/MS analysis. For Rat #1, an aliquot containing 25% desalted enriched phosphopeptides from Solution 1 and 2 were analyzed separately by HPLC-ESI-MS/MS 1st, followed by the analysis of the remaining 75% of the desalted phosphopeptides, respectively. For Rat #2 and #3, the entire desalted phosphopeptides from Solution 1 and 2 were analyzed separately by HPLC-ESI-MS/MS. Therefore, total 8 HPLC-ESI-MS/MS analyses were performed (4 for Rat #1 with 2 for Solution 1 and 2 for solution 2, 2 for Rat #2, and 2 for Rat #3). 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 Paradigm MS4 micro HPLC (Michrom BioResources, Auburn, CA) with a PicoFrit™ column (New Objective; 75 µm i.d., packed with ProteoPep™ II C18 material, 300 Å). 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, a hold of 5 min, and then a step to 80%, and a hold of 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, high mass accuracy data.

2.6. 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.RAT.v3.59 database (39,866 entries, <http://www.ebi.ac.uk/IPI/>) using Mascot Daemon (Matrix Science, London, UK; version 2.2). The false discovery rate was determined by selecting the option to search the decoy randomized database. Cross-correlation of Mascot search results with X! Tandem was accomplished with Scaffold (Proteome Software, Portland, OR). The search parameters were: 10 ppm mass tolerance for precursor ion masses and 0.5 Da for product ion masses; digestion with trypsin; a maximum of two missed tryptic cleavages; variable modifications of oxidation of methionine and phosphorylation of serine, threonine and tyrosine; and fixed modification of carbamidomethylation. Probability assessment of peptide assignments and protein identifications were made through use of Scaffold (version Scaffold_3_00_08, 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. Phosphorylation sites were located using Scaffold PTM (version 1.0.3, Proteome Software, Portland, OR, USA), a program based on Ascore algorithm [31, 32]. Sites with Ascores 13 ($P \leq 0.05$) were considered confidently localized [31, 32]. Manual inspection of the MS/MS spectra was performed to further confirm the results. If a peptide

has 2 or more mono phosphorylated isoforms identified by Scaffold 3 but each site with Ascore <13, manual inspection of the MS/MS spectra was performed to localize the phosphorylation site. If less than 2 unique fragment ions could be detected in the MS/Ms spectrum for each site, these sites will be classified as ambiguous and will be only counted as one site. If a peptide has a serine or threonine phosphorylation site identified, manual inspection of the MS/MS spectra was performed to confirm the phosphorylation site since pTyr-IP was utilized to enrich tyrosine phosphorylated peptides. Tyrosine kinases were predicted with a specificity of 90% using a web tool, KinasePhos (<http://kinasephos.mbc.nctu.edu.tw/>) [33, 34].

3. RESULTS

3.1. Rat skeletal muscle tyrosine phosphorylation

We performed pTyr-IP against tyrosine phosphorylated peptides from 10 mg of skeletal muscle lysates from 3 rats on a regular chow diet (Figure 1). HPLC-ESI-MS/MS analysis revealed 71, 63, and 77 non-redundant phosphorylation sites (with 95% confidence as assessed by Mascot and Scaffold analysis) from rats 1-3, respectively. For each identified phosphopeptide sequence, we have included the following in Supplemental Table 1: modifications, flank residues, precursor mass, charge and mass error observed, and the best Mascot score. Manual inspection of tandem MS spectra was performed to confirm phosphorylation site localization. Total number of distinctly localized pTyr sites from the three rats is 87. In addition, 4 ambiguous tyrosine phosphorylation sites were identified. 16 serine and 9 threonine localized phosphorylation sites were also detected. The phosphoserine/phosphothreonine/phosphotyrosine ratio of the present study was ~2:1:10 compared to ~18:4:1 in our previous work, using phosphopeptide enrichment with SCX and TiO₂ to characterize the human skeletal muscle phosphoproteome. These results agree with the literature that pTyr-IP prior to HPLC-ESI-MS/MS is a more efficient approach for tyrosine phosphoproteome studies [16, 17, 23, 25-30].

The 87 distinctly localized *in vivo* pTyr sites were localized in 46 proteins (Table 1). The false discovery rate, as assessed by Mascot searching of a randomized database, was 3% at the peptide level. Table 1 lists the identified isoforms of proteins defined as the 'canonical' sequence by UniProtKB/Swiss-prot. Out of the 87 distinctly localized pTyr sites, 56 have been reported in the four large phosphorylation site databases phosphosite.org, phospho.elm.eu.org, www.uniprot.org, and www.phosida.com (Table 1). Thus, 31 pTyr sites appear to be novel. Tandem mass spectra for three sites that were not reported in the 4 phosphorylation sites databases are included as Figure 2 and Supplemental Figure 1.

It is noted that in total, 942 unique peptides assigned to 182 unique proteins were identified in the experiments, including 794 non-phosphorylated peptides and 148 phosphopeptides. Among the unique phosphopeptides, 129 contain phosphorylated tyrosine residues, which correspond to 87 distinctly localized *in vivo* pTyr sites localized in 46 proteins as mentioned above.

3.2. Gene ontology annotation and functional classification

Gene ontology annotation (GO) and literature search revealed that 39 tyrosine phosphoproteins could be assigned to the cytoplasm, 13 to the nucleus, 8 to the membrane, 8 to the cytoskeleton, and 5 to the mitochondrion (Figure 3). Notably, some proteins can be assigned to multiple GO terms.

Closer examination of the protein functions revealed that 17 distinctly localized pTyr sites were identified in 8 of 13 major enzymes of the glycolytic pathway, while additional 9 distinctly localized pTyr sites were identified in 3 major enzymes involved in glycogen

metabolism and 3 signaling proteins known to regulate these enzymes (Figure 4). Another major part (40%) of the pTyr sites were found in sarcomeric proteins including several sites in the major myosin isoforms in mammalian skeletal muscle and the giant muscle protein, titin. We also identified tyrosine phosphorylation of proteins involved in phosphocreatine resynthesis, Ca^{2+} -homeostasis and MAP kinase signaling. Interestingly, we identified tyrosine phosphorylation of STAT5B, which has been shown to regulate skeletal muscle growth [11] (Table 1).

3.3. Potential kinases for identified tyrosine phosphorylation sites

Using KinasePhos [33, 34], we have predicted potential kinases for identified tyrosine phosphorylation sites (Supplemental Table 2). The number of distinct tyrosine phosphorylation sites by predicted kinase family for all phosphoproteins is shown in Figure 5. The potential tyrosine kinases include Insulin receptor (INSR), Epidermal growth factor receptor (EGFR), Tyrosine kinase Src (Src), Spleen Tyrosine kinase (Syk), Abelson murine leukemia virus oncoprotein (Ab1). Insulin receptor is the predicted kinase for 37 of the tyrosine sites identified, such as Tyr191 and Tyr195 in ADP/ATP translocase 1, Tyr44 in β -enolase, Tyr39, Tyr125, and Tyr140 in creatine kinase M-type, Tyr141 in elongation factor 1, Tyr44 in glycogen synthase, Tyr699 in Signal transducer and activator of transcription 5B. More experiments will be needed to verify whether these kinases indeed have the ability to phosphorylate these phosphorylation sites.

4. Discussions

4.1. pTyr-IP and rat skeletal muscle tyrosine phosphoproteome

Elucidating the role of tyrosine protein phosphorylation in normal skeletal muscle and its abnormalities in skeletal muscle disorders has been limited due to insufficient knowledge of protein tyrosine phosphorylation sites. Furthermore, identification of the tyrosine phosphorylation sites in mammalian skeletal muscle *in vivo* has been challenging due to technical limitations. Using a combination of phosphopeptide enrichment technique, pTyr-IP, with HPLC-ESI-MS/MS, the present study provides the first large-scale tyrosine phosphorylation study of rat skeletal muscle *in vivo* using 10mg of lysate proteins from relatively small tissue samples, a muscle biopsy of approximately 100 mg wet weight. We identified 87 distinctly localized and 4 ambiguous pTyr sites in 46 proteins from 3 rats. Of these distinctly localized ones, 56 sites have previously been reported in four large protein phosphorylation site databases from various cell lines or species, while 31 appear to be novel. Even though the absolute number of phosphorylation sites identified is low when compared to large-scale phosphoproteomic studies of human cell lines [16, 22-27], it is the first global analysis of pTyr sites in mammalian skeletal muscle. In addition, since estimated tyrosine phosphorylation accounts for less than 1% of the phosphoproteome [1], the finding of 87 pTyr sites in our study suggest that a total of about 8700 phosphorylation sites in rat skeletal muscle.

The majority of tyrosine phosphorylated proteins identified in skeletal muscle were high abundant proteins, such as sarcomeric proteins and enzymes involved in glycolysis, glycogen metabolism, Ca^{2+} -homeostasis and the phosphocreatine shuttle. However, we also identified many known as well as new pTyr sites on low abundant signaling intermediates, such as several MAP kinases, PTPN11, GSK3, DYRK1, STAT5B, and GYS1. This indicates that our phosphopeptide tyrosine immunoprecipitation protocol may be suitable for targeted phosphoproteomic studies of tyrosine phosphorylated signaling events in skeletal muscle, such as in response to insulin stimulation or exercise.

In addition to detecting tyrosine phosphorylation sites, 16 serine and 9 threonine phosphorylation sites were identified. Among the 16 serine phosphorylation sites, 1 was

detected in a doubly phosphorylated peptide with both pY279 and pS282 in GSK3A; 9 were detected in double or triple serine/threonine phosphorylated peptides, including HRC (pS466, pS471), HRC (pS466, pS471 and pT476), HRC (S549, S550), PDAP1 (S60, S63), PDHA1 (S293, S300), TTN (T32001, S32003); and 6 were detected in peptides with single phosphorylated serine residues. For the 9 threonine phosphorylation sites, 4 were detected in doubly phosphorylated peptides containing a tyrosine phosphorylation site, including MAPK1 (T183, Y185), MAPK3 (T203, Y205), MAPK12 (T183, Y185), and MAPK14 (T180, Y182); 2 were detected in double or triple serine/threonine phosphorylated peptides, including HRC (pS466, pS471 and pT476) and TTN (T32001, S32003); and 3 were detected in peptides with single phosphorylated threonine residues. These results along with the 794 non-phosphorylated peptides identified in the study indicated that even though pTyr IP is relatively specific, non-specific binding might still present a problem. Nonetheless, in our previous study to characterize human skeletal muscle proteome [35], 7657 unique peptides in 954 proteins were detected from muscle biopsies, while only 4 pTyr sites in 4 proteins were detected. In addition, as discussed before, we previously identified 13 pTyr sites in 9 proteins out of 306 phosphorylation sites in 127 proteins in human skeletal muscle [19]. On the other hand, in the present study, 87 pTyr sites were identified in 46 proteins out of 182 proteins, indicating pTyr-IP is an efficient way to enrich tyrosine phosphorylated peptides.

4.2. Tyrosine phosphorylation of glycolytic enzymes

An important finding of the present study is that the majority of glycolytic enzymes are tyrosine phosphorylated in rat skeletal muscle *in vivo*. 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 or by transformation of normal cells into cancer cells [16, 36-40]. It has been proposed that tyrosine phosphorylation of glycolytic enzymes is implicated in the switch from oxidative phosphorylation to aerobic glycolysis (Warburg effect) that is important for cancer cell and tumor growth [40]. This suggests that increased tyrosine phosphorylation of glycolytic enzymes in skeletal muscle could contribute to the decreased ratio between glycolytic and oxidative enzyme activities observed in insulin resistant conditions such as obesity and T2D [41, 42]. Moreover, tyrosine phosphorylation of GAPDH in response to insulin may contribute to the interaction of GAPDH with GLUT4, which has been proposed to regulate the intrinsic activity of GLUT4 [43]. In support of this, it was recently demonstrated that insulin increased tyrosine phosphorylation of GAPDH at Tyr316 and of ENO1/3 at Tyr44 by more than 1.3 fold in human adipocytes [17]. These findings also support the notion that insulin, similar to other growth factors, stimulates glycolysis by tyrosine phosphorylation of glycolytic enzymes. Further studies are needed to establish the role of tyrosine phosphorylation of glycolytic enzymes in metabolic homeostasis in normal and diseased human skeletal muscle.

4.3. Tyrosine phosphorylation of glycogen metabolism enzymes

In addition to glycolytic enzymes, we found several pTyr-sites on major enzymes involved in glycogen metabolism, including a phosphorylation site at Tyr44 on muscle glycogen synthase (GYS1) and constitutive activation-loop autophosphorylation of two serine/threonine kinases, GSK3A (Tyr279) and DYRK1A (Tyr321) [44, 45], known to inactivate GYS1 by phosphorylation at specific C-terminal Ser residues [7, 46], respectively. Insulin stimulates GYS1 by dephosphorylation of several serine residues, and this effect is impaired in insulin resistant conditions [46-48]. The Tyr44 residue of GYS1 is located close to the reported UDP-glucose binding site of GYS1 at Lys39 in rat skeletal muscle [49], suggesting that phosphorylation at pTyr44 may modulate the binding between GYS1 and UDP-glucose. It is tempting to speculate that hormonal factors such as insulin as well as muscle contraction may regulate phosphorylation of Tyr44, which will contribute to the regulation

of glycogen synthesis. Muscle contraction stimulates phosphorylase kinase which in turn activates glycogen phosphorylase (PYGM) by phosphorylation of Ser15. In the present study, we found a novel pTyr-site at Tyr350 on the catalytic gamma subunit of phosphorylase kinase (PHKG1) and several pTyr-sites on PYGM in rats in the resting, basal state, suggesting that tyrosine phosphorylation may contribute to keep these enzymes in an inactive state under these conditions. Further studies are warranted to determine the potential role of these pTyr-sites on enzymes involved in glycogen synthesis and glycogenolysis in response to various physiological stimuli and pathophysiological conditions.

4.4. Tyrosine phosphorylation on mitochondrial proteins

In the present study, we identified three pTyr-sites on two mitochondrial proteins, ADP/ATP translocase 1 (SLC25A4/ANT1) and the mitochondrial creatine kinase (CKMT2) in whole muscle cell lysates using pTyr-IP and MS/MS. Recently, phosphorylation of ANT1 at Tyr195 was suggested to be critical for mitochondrial respiration in rat heart muscle [50] and together with the cytosolic and mitochondrial isoenzymes of creatine kinase (CKM and CKMT2) ANT1 regulate the phosphocreatine/creatine pool in muscle cells by resynthesizing phosphocreatine from mitochondrial ATP synthesis [51]. This suggests that tyrosine phosphorylation of ANT1, CKMT2 and CKM could regulate re-synthesis of ATP after muscle work in mammalian skeletal muscle. In a recent phosphoproteomic study of isolated mitochondria from human skeletal muscle, 16 pTyr-sites on 12 mitochondrial proteins were reported [52]. Consistently, numerous tyrosine phosphorylated mitochondrial proteins have been discovered within the last decade using a variety of proteomic and phosphoproteomic approaches [53, 54]. We therefore assume that a more comprehensive identification of the mitochondrial tyrosine phosphoproteome in mammalian skeletal muscle than that observed by Zhao et al [52] can be acquired by the isolation of mitochondria prior to immunoprecipitation of phosphotyrosine peptides and subsequent HPLC-ESI-MS/MS.

4.5. Tyrosine phosphorylation of sarcomeric proteins

The sarcomere is the basic functional unit in striated muscle contraction. Using the phosphoproteomic approach, we identified 33 distinctly localized pTyr-sites on muscle- and fiber-type specific isoforms of myosins and thin filament proteins (α -actin and tropomyosins), the giant muscle protein, titin, the fast-fiber specific Z-disc protein, α -actinin-3, as well as 5 pTyr-sites on proteins involved in excitation-contraction coupling (the Ca^{2+} -cycle). Information about tyrosine phosphorylation of sarcomeric proteins in adult mammalian skeletal muscle is scarce, but previous studies have suggested that tyrosine phosphorylation of myosin heavy chains is mediated by insulin [55], and may be involved in skeletal muscle differentiation (reorganization) [56]. Here, we report pTyr-sites on all 3 major adult heavy myosin chains (MYH1, MYH2 and MYH7), in resting skeletal muscle. However, further studies are needed to address the potential role of these pTyr-sites, and whether they regulate the actomyosin interaction and myosin ATPase activity *in vivo*.

4.6. Comparison to pTyr-IP in quantitative phosphoproteomic studies of mouse adipocytes

Recently, Schmelzle et al. [17] studied insulin-induced tyrosine phosphorylation in mouse adipocytes using a protocol very similar to that in the present study. By combining anti-phosphotyrosine peptide immunoprecipitation and immobilized metal affinity chromatography (IMAC) with MS/MS, they reported relative quantification of 122 pTyr-sites on 89 proteins in 3T3-L1 adipocytes stimulated with insulin for 0, 5, 15, and 45 min [17]. Strikingly, several of the same pTyr-sites were identified in our study. This included pTyr-sites showing at least a 3-fold increase in response to insulin such as MAPK3/Erk1 (Tyr205), MAPK1/Erk2 (Tyr185), ENO1/3 (Tyr44), GAPDH (Tyr316), and MAP kinase p38 α (Tyr182). In addition, several proteins with pTyr-sites not responding to insulin were identified in both studies including DYRK1A (Ty321), EEF1A2 (Tyr141), GSK3A

(Tyr279), HIPK3 (Tyr359), PRPF4B/PRP4 (Tyr849), PXN (Tyr118) and SHP-2/PTPN11 (Tyr62). The protein-tyrosine phosphatase 2 (PTPN11) is required for insulin activation of MAPK1/Erk2 by increasing phosphorylation of Thr183 and Tyr185 [57]. Taken together, these findings demonstrate that pTyr-IP may be useful in quantitative phosphoproteomic studies of low-abundant enzymes regulated by insulin and growth factor signaling.

5. Conclusions

Using pTyr-IP followed by HPLC-ESI-MS/MS, we identified 87 distinctly localized (31 novel) in 46 rat skeletal muscle proteins, the largest catalogue of mammalian skeletal muscle tyrosine phosphorylation sites to date. The identified tyrosine phosphorylated proteins includes most major enzymes in the glycolytic pathway, enzymes regulating glycogen metabolism, proteins involved in Ca^{2+} -homeostasis and phosphocreatine re-synthesis, as well as MAP kinases and mitochondrial proteins. These results provide multiple novel protein pTyr-sites for the investigation of human skeletal muscle in conditions of health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Identified 87 distinctly localized tyrosine phosphorylation sites (31 were novel) in 46 muscle proteins from a relative small rat muscle biopsies
- Demonstrated the feasibility of using immunoprecipitation of phosphotyrosine peptides to improve the discovery of tyrosine phosphorylation in muscle
- Provide the largest catalogue of mammalian skeletal muscle tyrosine phosphorylation sites to date
- Provide novel targets for the investigation of human skeletal muscle phosphoproteins in various disease states

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

b-ions 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
 G-T-G-G-V-D-T-A-A-V-A-D-V-pY-D-I-S-N-I-D-R
 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 y-ions

Ions	Theoretical	Experimental	Difference	Ions	Theoretical	Experimental	Difference
y3	403.23	403.33	0.10	y8	1075.45	1075.49	0.05
b6 [#]	469.20	469.29	0.09	y9	1174.51	1174.51	0.00
y4	517.27	517.29	0.02	y10	1289.54	1289.61	0.06
y5	604.30	604.36	0.05	y11	1360.58	1360.59	0.02
b8 [#]	641.29	641.50	0.21	y12	1459.65	1459.67	0.02
b9 [#]	712.33	712.35	0.03	b15	1472.59	1472.59	-0.01
y6	717.39	717.36	-0.03	y13	1530.68	1530.86	0.18
b9	730.34	730.42	0.09	b16 [#]	1567.66	1567.67	0.01
b10 [#]	811.39	811.35	-0.04	b16	1585.68	1585.60	-0.07
b10	829.41	829.36	-0.05	y14	1601.72	1601.78	0.06
y7	832.42	832.43	0.01	y15	1702.77	1702.64	-0.13
b11 [#]	882.43	882.43	0.00	y16	1817.80	1817.83	0.04
b12	1015.47	1015.45	-0.02	b19	1899.84	1899.74	-0.10

Figure 2.

Representative MS/MS spectrum for a novel phosphorylated peptide ₃₅₅GTGGVDTAADVpYDISNIDR₃₇₅ of sarcomeric mitochondrial creatine kinase. The ions corresponding to b₉, b₁₀, b₁₂ and y₅, y₆, y₇ are consistent with phosphorylation on Tyr368.

Figure 3. Subcellular location of identified phosphoproteins in rat skeletal muscle based on gene ontology annotation and literature search.

(A) Glycolytic pathway

(B) Glycogen metabolism

(C) Muscle glycogen synthase (GYS1)

Figure 4.

Tyrosine phosphorylation of enzymes involved in glycolysis and glycogen metabolism. Isoforms and/or subunits of enzymes involved in (A) glycolysis, (B) glycogen metabolism, and (C) regulation of muscle glycogen synthase (GYS1). Tyrosine phosphorylated proteins are shown in *yellow boxes*, and the number of identified tyrosine phosphorylation sites (pY) are indicated by red circles. Number of serine (Ser) residues on GYS1 indicates known Ser phosphorylation sites with their classical number of site given below the bar. AMPK, AMP-activated kinase; CaM-KII, calcium-calmodulin-dependent kinase II; CK1, casein kinase 1; CK2, casein kinase 2; DYRK1A, dual specificity tyrosine-phosphorylation-regulated kinase 1A; GSK3A, glycogen synthase kinase a; PHK, phosphorylase kinase; PKA, protein kinase A; PKC, protein kinase C.

Figure 5. Number of distinct pY sites by potential kinase family for all phosphoproteins predicted by KinasePhos [33]. Insulin receptor (INSR), Epidermal growth factor receptor (EGFR), Tyrosine kinase Src (Src), Spleen Tyrosine kinase (Syk), Abelson murine leukemia virus oncoprotein (Ab1).

Table 1Phosphorylation sites identified *in vivo* in rat muscle biopsy using pTyr-IP followed by HPLC-ESI-MS/MS.

Gene	Protein	Phosphorylation Sites
<i>Glycolysis</i>		
FBP2	Fructose-1,6-bisphosphatase 2	Y216
ALDOA	Fructose-bisphosphate aldolase A	Y223
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Y316, Y328
PGK1	Phosphoglycerate kinase 1	Y76, Y196
PGAM2	Phosphoglycerate mutase 2	Y92, Y132 *, Y133
ENO1	α -enolase	Y25, Y44
ENO3	β -enolase	Y44 *, Y131, Y252 *
PKM2	Pyruvate kinase isozymes M1/M2	Y148
LDHA	L-lactate dehydrogenase A chain	Y127, Y239
<i>Glycogen metabolism</i>		
GYS1	Glycogen synthase, muscle	Y44 *
PYGM	Glycogen phosphorylase, muscle form	Y76 *, Y204 *, Y227 *, Y473, Y525 *, Y732 *, Y733
PGM1	Phosphoglucomutase 1	Y521 *
GSK3A	Glycogen synthase kinase 3 α	Y279, S282
DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A	Y321
PHKG1	Phosphorylase b kinase gamma catalytic chain	Y350 *
<i>Phosphocreatine shuttle</i>		
CKM	Creatine kinase M-type	Y39 *, Y125, Y140 *, Y174
CKMT2	Creatine kinase, sarcomeric mitochondrial precursor	Y368 *
SLC25A4	ADP/ATP translocase 1 (ANT1)	Y191, Y195
<i>Ca²⁺- regulated/binding proteins</i>		
ATP2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	Y15 *, Y527
ANXA6	Annexin A6	Y609
CASQ1	Calsequestrin 1 (fragment)	Y9 *
HRC	Sarcoplasmic reticulum histidine-rich calcium-binding protein	S228 *, S378 *, S466 *, S471 *, T476, S549, S550
SRL	Sarcalumenin (98 kDa protein)	Y484 *
<i>MAP-kinase signaling</i>		
MAPK1	Mitogen-activated protein kinase 1 (Erk2)	T183, Y185
MAPK3	Mitogen-activated protein kinase 3 (Erk1)	T203, Y205
MAPK12	Mitogen-activated protein kinase 12 (MAP kinase p38 gamma)	T183, Y185
MAPK14	Mitogen-activated protein kinase 14 (MAP kinase p38 α)	T180, Y182
PTPN11	Tyrosine-protein phosphatase non-receptor type 11 (SHPTP2)	Y62

Gene	Protein	Phosphorylation Sites
<i>Sarcomeric function</i>		
ACTA1	α -Actin, skeletal muscle	Y55
TPM1	Tropomyosin 1	Y162, Y261
TPM2	Tropomyosin 2	Y60 [*] , Y162, Y261
MYH1	Myosin-1	Y622 [*] , Y1294 [*] , Y1495
MYH2	Myosin-2	Y1859
MYH4	Myosin-4	Y389, Y424 [*] , Y820 [*] , Y1351 [*] , Y1464, Y1492 [*] , S1495 [*] , Y1856
MYH7	Myosin-7	Y410, Y1308 [*] , Y1375, Y1852
MYLPF	Myosin regulatory light chain 2, skeletal muscle	Y158
ACTN3	α -actinin-3	Y850 [*]
TTN	Titin	Y11721/T11723 [*] , Y18039 [*] , Y18936 [*] , Y21990, Y23841 [*] , Y24117, Y25200, S26266 [*] /Y26267 [*] /T26270 [*] , Y26279, Y28244/T28245 [*] , Y28450 [*] , Y29539 [*] , Y30067 [*] , Y30569 [*] /Y30570 [*] , T32001, S32003
<i>Miscellaneous</i>		
DLG5	Discs, large homolog 5	S1367 [*]
EEF1A2	Elongation factor 1- α 2	Y141, T142
GPR108	Protein GPR108	T42 [*]
HIPK3	Homeodomain interacting protein kinase 3	Y359
HSPA8	Heat shock cognate 71kDa protein	Y107
LKAP	Limkain-b1	S71 [*]
MDH1	Malate dehydrogenase, cytoplasmic	Y119
PDAP1	28 kDa heat-and acid-stable phosphoprotein	S60, S63
PDHA1	Pyruvate dehydrogenase E1 component subunit α , somatic form	S293, S300
PRPF4B	Serine/threonine-protein kinase PRP4 homolog	Y849
PXN	Paxillin (64 kDa protein, IPI00363651, IPI00471807)	Y114
RPS27L	40S ribosomal protein S27-like protein	Y31
STAT5B	Signal transducer and activator of transcription 5B	Y699
VPS13C	Vacuolar protein sorting 13C protein, isoform 1A	T1434
-----	64 kDa protein, IPI00191437	S6 [*]

Ambiguous sites were indicated by “/”.

* Novel phosphorylation sites.