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Phosphoproteome Analysis of Functional Mitochondria Isolated from Resting Human Muscle Reveals Extensive Phosphorylation of Inner Membrane Protein Complexes and Enzymes*[S]

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Mitochondria play a central role in energy metabolism and cellular survival, and consequently mitochondrial dysfunction is associated with a number of human pathologies. Reversible protein phosphorylation emerges as a central mechanism in the regulation of several mitochondrial processes. In skeletal muscle, mitochondrial dysfunction is linked to insulin resistance in humans with obesity and type 2 diabetes. We performed a phosphoproteomics study of functional mitochondria isolated from human muscle biopsies with the aim to obtain a comprehensive overview of mitochondrial phosphoproteins. Combining an efficient mitochondrial isolation protocol with several different phosphopeptide enrichment techniques and LC-MS/MS, we identified 155 distinct phosphorylation sites in 77 mitochondrial phosphoproteins, including 116 phosphoserine, 23 phosphothreonine, and 16 phosphotyrosine residues. The relatively high number of phosphotyrosine residues suggests an important role for tyrosine phosphorylation in mitochondrial signaling. Many of the mitochondrial phosphoproteins are involved in oxidative phosphorylation, tricarboxylic acid cycle, and lipid metabolism, *i.e.* processes proposed to be involved in insulin resistance. We also assigned phosphorylation sites in mitochondrial proteins involved in amino acid degradation, importers and transporters, calcium homeostasis, and apoptosis. Bioinformatics analysis of kinase motifs revealed that many of these mitochondrial phosphoproteins are substrates for protein kinase A, protein kinase C, casein kinase II, and DNA-dependent protein kinase. Our results demonstrate the feasibility of performing phosphoproteome analysis of organelles isolated from human tissue and provide novel targets for functional studies of reversible phosphorylation in mitochondria. Future comparative phosphoproteome analysis of mitochondria from healthy and diseased individuals will provide insights into the role of abnormal phosphorylation in pathologies, such as type 2 diabetes. ***Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.000299, 1–14, 2011.***

Mitochondria are the primary energy-generating systems in eukaryotes. They play a crucial role in oxidative metabolism, including carbohydrate metabolism, fatty acid oxidation, and urea cycle, as well as in calcium signaling and apoptosis (1, 2). Mitochondrial dysfunction is centrally involved in a number of human pathologies, such as type 2 diabetes, Parkinson disease, and cancer (3). The most prevalent form of cellular protein post-translational modifications (PTMs),[1] reversible phosphorylation (4–6), is emerging as a central mechanism in the regulation of mitochondrial functions (7, 8). The steadily increasing numbers of reported mitochondrial kinases, phosphatases, and phosphoproteins imply an important role of protein phosphorylation in different mitochondrial processes (9–11).

Mass spectrometry (MS)-based proteome analysis is a powerful tool for global profiling of proteins and their PTMs, including protein phosphorylation (12, 13). A variety of proteomics techniques have been developed for specific enrichment of phosphorylated proteins and peptides and for phosphopeptide-specific data acquisition techniques at the MS level (14). Enrichment methods based on affinity chromatography, such as titanium dioxide (TiO_2) (15–17), zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) (18), im-

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[1] The abbreviations used are: PTM, post-translational modification; TiO_2, titanium dioxide; ZIC-HILIC, zwitterionic hydrophilic interaction chromatography; CPP, calcium phosphate precipitation; LTQ, linear ion trap; MSA, multistage activation; FA, formic acid; IPI, International Protein Index; VDAC, voltage-dependent anion channel; CKII, casein kinase II; DNAPK, DNA-dependent protein kinase.

mobilized metal affinity chromatography (IMAC) (19, 20), and ion exchange chromatography (strong anion exchange and strong cation exchange) (21, 22), have shown high efficiencies for enrichment of phosphopeptides (14). Recently, we demonstrated that calcium phosphate precipitation (CPP) is highly effective for enriching phosphopeptides (23). It is now generally accepted that no single method is comprehensive, but combinations of different enrichment methods produce distinct overlapping phosphopeptide data sets to enhance the overall results in phosphoproteome analysis (24, 25). Phosphopeptide sequencing by mass spectrometry has seen tremendous advances during the last decade (26). For example, MS/MS product ion scanning, multistage activation, and precursor ion scanning are effective methods for identifying serine (Ser), threonine (Thr), and tyrosine (Tyr) phosphorylated peptides (14, 26).

A "complete" mammalian mitochondrial proteome was reported by Mootha and co-workers (27) and included 1098 proteins. The mitochondrial phosphoproteome has been characterized in a series of studies, including yeast, mouse and rat liver, porcine heart, and plants (19, 28–31). To date, the largest data set by Deng *et al.* (30) identified 228 different phosphoproteins and 447 phosphorylation sites in rat liver mitochondria. However, the *in vivo* phosphoproteome of human mitochondria has not been determined. A comprehensive mitochondrial phosphoproteome is warranted for further elucidation of the largely unknown mechanisms by which protein phosphorylation modulates diverse mitochondrial functions.

The percutaneous muscle biopsy technique is an important tool in the diagnosis and management of human muscle disorders and has been widely used to investigate metabolism and various cellular and molecular processes in normal and abnormal human muscle, in particular the molecular mechanism underlying insulin resistance in obesity and type 2 diabetes (32). Skeletal muscle is rich in mitochondria and hence a good source for a comprehensive proteomics and functional analysis of mitochondria (32, 33).

The major aim of the present study was to obtain a comprehensive overview of site-specific phosphorylation of mitochondrial proteins in functionally intact mitochondria isolated from human skeletal muscle. Combining an efficient protocol for isolation of skeletal muscle mitochondria with several different state-of-the-art phosphopeptide enrichment methods and high performance LC-MS/MS, we identified 155 distinct phosphorylation sites in 77 mitochondrial phosphoproteins, many of which have not been reported before. We characterized this mitochondrial phosphoproteome by using bioinformatics tools to classify functional groups and functions, including kinase substrate motifs.

EXPERIMENTAL PROCEDURES

Materials

Pure water was obtained from a Milli-Q system (Millipore, Bedford, MA). Modified trypsin was from Promega (Madison, WI). POROS OLIGO R3 reversed phase material was from PerSeptive Biosystems (Framingham, MA). GELoader tips were from Eppendorf (Hamburg, Germany). 3M Empore™ C_8 disks were from 3M Bioanalytical Technologies (St. Paul, MN). TiO_2 resin (5 μm) was obtained from a disassembled titanium dioxide cartridge purchased from GL Sciences Inc. (Tokyo, Japan). All other reagents and solvents were of the highest commercial quality and were used without further purification.

Subjects and Muscle Biopsies

Skeletal muscle samples were obtained from five healthy, nonobese volunteers (age, 28–54 years; body mass index, 22.8–27.8 kg/m^2). The muscle biopsies from these individuals were named Subject 1, Subject 2, Subject 3, Subject 4 and Subject 5, respectively. The volunteers had no family history of type 2 diabetes and were not taking any medication. Informed consent was obtained from all individuals before participation. The study was approved by the local ethics committee and was performed in accordance with the Helsinki Declaration.

A percutaneous needle biopsy was taken under local anesthesia from the vastus lateralis muscle using a modified Bergström needle with suction while the subject rested in a supine position (34). Muscle samples were immediately blotted free of blood, fat, and connective tissue and used for isolation of mitochondria as described below.

Preparation of Mitochondria

Isolation of human skeletal muscle mitochondria was performed as described previously (35, 36). When using this technique, both subsarcolemma and intermyofibrillar mitochondria are isolated. Briefly, the muscle samples were weighed, and pieces of 100 mg from each muscle sample were cut with scissors in 1 ml of isolation medium (100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 mM KH_2PO_4, 0.1 mM EGTA, 0.2% BSA, 1:100 protein phosphatase inhibitor mixtures 1 and 2 (Sigma), protease inhibitor tablet (Roche Applied Science), 2 mM sodium pervanadate, 1 mM EDTA, pH 7.4). Muscle pieces were rinsed thoroughly in the isolation medium and transferred to 1 ml of isolation medium supplemented with 0.2 mg/ml nagarse (Sigma). After 2 min, muscles were homogenized using a motor-driven Plexiglas homogenizer. After homogenization, 3 ml of isolation medium was added to the homogenate and centrifuged at 700 $\times$ *g* for 10 min. The supernatant was decanted and centrifuged at 10,000 $\times$ *g* for 10 min. The resulting pellet containing the mitochondria was washed, resuspended in 650 μl of isolation medium, and centrifuged at 7000 $\times$ *g* for 4 min. The final mitochondrial pellet was resuspended in suspension medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris, 0.1 mM EGTA, pH 7.4 at a final protein concentration of about 20 mg/ml. All procedures were carried out at 0–4 °C.

Extraction and Digestion of Mitochondrial Proteins

The mitochondrial suspension was centrifuged at 10,000 $\times$ *g* for 10 min. The pellet was resuspended in 200 μl of lysis buffer containing 7 M urea, 2 M thiourea, 1:100 protein phosphatase inhibitor mixtures 1 and 2 (Sigma). After sonication for 3 min (5-s intervals for every 2-s sonication), sonicated lysate was centrifuged at 20,000 $\times$ *g* for 20 min. The protein concentration was determined by the Bradford assay. The protein solution was reduced with DTT (5 mM) at 37 °C for 45 min, alkylated with iodoacetamide (15 mM) at room temperature for 45 min, and then digested with Lys-C (1:30) at room temperature for 4 h. After 6-fold dilution with H_2O, the solution was subsequently digested overnight with trypsin (1:50) at 37 °C. Note that the pH value should be around 8.0 and was checked before each digestion step. 5% $NH_3{\cdot}H_2O$ was used to adjust the pH. 200 μg of mitochondrial protein preparation was used for each phosphopeptide enrichment approach.

TiO_2 Enrichment

Microcolumn Mode—Phosphopeptides were enriched using TiO_2 solid phase extraction in a microcolumn setup as described by Thingholm *et al.* (16). A small C_8 plug (3M C_8 disk) was made using an HPLC syringe (Microlab Aarhus) and placed at the constricted end of the GELoader tip. The TiO_2 material in 100% ACN was packed on top of the C_8 plug. The peptide solution in the loading buffer (80% ACN, 5% TFA, 1 M glycolic acid) was loaded onto the microcolumn. After washing with 20 μl of loading buffer and 10 μl of washing buffer (80% ACN, 1% TFA), the bound peptides were eluted with 20 μl of $NH_3{\cdot}H_2O$, pH 10.5. The elution was acidified with 2 μl of 100% formic acid and desalted by R3 microcolumn prior to MS analysis.

Batch Mode—For batch mode phosphopeptide enrichment, TiO_2 material was weighed and put into an Eppendorf tube (0.4 mg/100 μg of protein). The sample was diluted five times with loading buffer (80% ACN, 5% TFA, 1 M glycolic acid) and mixed with TiO_2 material in the tube for 10 min. After spinning down, the supernatant was removed. The resin was washed with 50 μl of loading buffer, 50 μl of washing buffer 1 (80% ACN, 1% TFA), 50 μl of washing buffer 2 (20% ACN, 0.1% TFA), and 50 μl of water. Then the resin was mixed with 100 μl of elution buffer ($NH_3{\cdot}H_2O$, pH 10.5). The supernatant containing the phosphopeptides was collected. The elution was acidified with 100% formic acid and desalted by R3 prior to MS analysis.

ZIC-HILIC Microcolumn

ZIC-HILIC material was from Merck/SeQuant (Umeå, Sweden). HILIC medium (10 μm) was washed three times in methanol, three times in H_2O, and finally suspended in methanol. The off-line HILIC enrichment of phosphopeptides in this study was inspired by the on-line method reported by McNulty and Annan (18). ZIC-HILIC resin was packed into GELoader tips (about 7-mm column height). Columns were washed and equilibrated with loading buffer (95% ACN, 0.1% TFA). Samples were loaded onto HILIC microcolumns in 20 μl of loading buffer. After washing with loading buffer, bound phosphopeptides were stepwise eluted with elution solvent containing 0.5% TFA and decreasing ACN concentrations. In total, six fractions were collected, dried in a vacuum centrifuge, and redissolved in TiO_2 loading buffer for further phosphopeptide enrichment.

Calcium Phosphate Precipitation

CPP was performed according to Zhang *et al.* (23) with minor modifications. The total volume of the peptide solution was adjusted to 50 μl. 2 μl of 0.5 M Na_2HPO_4 and 2 μl of 2 M NH3·H_2O were added and mixed followed by the addition of 2 μl of 2 M $CaCl_2$. It should be noted that the pH value of the buffer prior to adding $CaCl_2$ should be around 10. The solution was vortexed and centrifuged at 20,000 $\times$ *g* for 10 min at room temperature. Subsequently, the supernatant was removed, and 60 μl of 80 mM $CaCl_2$ was applied to suspend and wash the pellet. After centrifugation as described above, the washing solution was removed, and the resulting pellet was redissolved in 20 μl of 5% formic acid, desalted by R3, and subjected to TiO_2 enrichment.

Peptide Desalting

GELoader tip microcolumns were packed with the POROS OLIGO R3 reversed phase resin (20 μm; Applied Biosystems). The length of microcolumns was made according to the sample amount. The columns were equilibrated with 5% formic acid. Samples were loaded onto the columns in 10 μl of 5% formic acid, washed with 20 μl of 5% formic acid, and eluted with 20 μl of 70% ACN, 5% formic acid. The elution was dried in vacuum and stored at -80 °C for later HPLC-ESI-MS/MS analysis. For subsequent analysis on TiO_2, the peptides were directly eluted onto the TiO_2 column with 80% ACN, 5% FA.

Data Acquisition

Capillary LC-MS/MS analyses were performed using an EasyLC system (Proxeon Biosystems/ThermoFisher, Odense, Denmark) interfaced to an LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher, Bremen, Germany). Samples were dissolved in Solvent A (0.5% FA) and loaded at a flow rate of 550 nl/min onto a custom-made 16-cm analytical column (100-μm inner diameter, 375-μm outer diameter, packed with Reprosil C_{18}, 3-μm reversed phase particles (Dr. Maisch GmbH)). Separation was then performed at a flow rate of 250 nl/min using a 50- or 100-min gradient of 0–34% Solvent B (90% ACN, 0.5% FA) into the nanoelectrospray ion source (Proxeon Biosystems). The LTQ-Orbitrap XL instrument was operated in a data-dependent MS/MS mode using multistage activation (MSA) (37). The peptide masses were measured by the Orbitrap, and up to five of the most intense peptides were selected and subjected to fragmentation using MSA in the linear ion trap (LTQ).

Database Search and Data Interpretation

The LC-MS/MS data were processed (smoothing, background subtraction, and centroiding) using Proteome Discoverer (Version 1.2, ThermoFisher) (supplemental Fig. 1). The processed LC-MS/MS data were submitted to database searching against the human sequence library in the International Protein Index (IPI) protein sequence database (IPI human; November 30, 2009; 90,807 sequences) using an in-house Mascot server (Version 2.2.04, Matrix Science Ltd., London, UK). Trypsin was chosen as the enzyme with a maximum of two missed cleavages allowed. *S*-Carbamidomethylcysteine was defined as a fixed modification. Partial modifications included oxidation (Met), *N*-acetylation (protein N terminus), phosphorylation (Ser, Thr, and Tyr), and intact phosphorylation (Ser, Thr, and Tyr). The MS and MS/MS results were searched with a peptide ion mass tolerance of 5 ppm and a fragment ion mass tolerance of $\pm$0.6 Da. A decoy database search was performed using a concatenated decoy human database (IPI human decoy; total number of sequences, 136,764) derived from the IPI human database. Only peptides that were identified as peptide rank 1 and with a peptide expectation value of $p < 0.05$ were considered for further analysis.

Candidate phosphopeptides that were identified with a Mascot score higher than 20 were considered for further interpretation by manual inspection of their respective MS/MS spectra. Verification was carried out based on the occurrence of consecutive y or b ions. The presence of a phosphate group and the site for phosphorylation assigned by Mascot were manually validated. The phosphorylated sites were assigned by the presence of a 69/167-Da distance between fragment ions for phosphoserine and an 83/181-Da distance for phosphothreonine. Phosphotyrosine peptides were validated by the observation of a mass increase of 80 Da to unmodified peptide and the appearance of the immonium ion at 216 Da in spectra when 216 Da is within the mass range. The modified sites were determined by the detection of a mass difference of 243 Da between fragment ions.

Annotation and classification of the identified proteins and phosphoproteins into mitochondrial and non-mitochondrial proteins were facilitated by using Protein Center (ThermoFisher Scientific, Odense, Denmark), gene ontology information, and the human MitoCarta list of 1013 mitochondrial genes by Pagliarini *et al.* (27). The IPI identities, gene names, and UniProt and Swiss-Prot identities of the identified mitochondrial proteins were also retrieved through Protein Center together with UniProt (http://www.uniprot.org/). Among the 545 proteins identified in the four different phosphopeptide enrichment preparations of isolated mitochondria, a total of 310 mitochondrial proteins were annotated as mitochondrial proteins by Protein Center and MitoCarta.

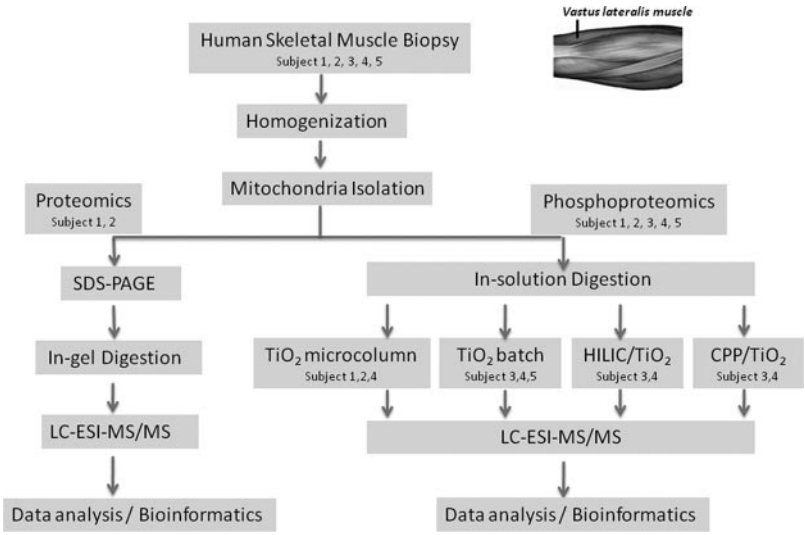

FIG. 1. **Flow chart for analysis of human *in vivo* skeletal muscle mitochondrial proteome and phosphoproteome.**

RESULTS

Isolation and Assessment of Mitochondria from Human Skeletal Muscle—We initially assessed the muscle tissue sampling and mitochondrial isolation methods using respiratory analysis and LC-MS/MS-based proteome analysis.

To verify a high purity and quality of the isolated muscle mitochondria, a respiratory analysis was conducted using a simple state 3 (ADP-activated)-state 4 (without ADP) transition with pyruvate and malate as substrates (35). The phosphate to oxygen ratio (ADP consumption divided by oxygen utilization) was 2.85 ± 0.01, and the respiratory control index (state 3 divided by state 4) was 15.4 ± 3.0, confirming that the isolated mitochondria were fully functional and that the methods used for tissue sampling and mitochondrial preparation were of high quality.

To assess the enrichment of mitochondrial proteins in our preparation of isolated mitochondria, the extracted proteins were identified by using SDS-PAGE separation followed by in-gel digestion and LC-MS/MS analysis of peptides (Fig. 1 and Table I). This large scale approach is preferable to Western blotting for a few mitochondrial reporter proteins because proteome analysis provides a better overview of the protein composition and protein functional classes.

Two proteomics experiments were carried out using 60 (Subject 1) and 70 μg (Subject 2) of isolated mitochondrial protein starting material, respectively (Fig. 1 and supplemental Table 1). In one experiment, 652 proteins were identified of which 375 (58%) were assigned as mitochondrial proteins by gene ontology analysis. The fraction of MS/MS spectra that originated from peptides from mitochondrial proteins was 72.4%. In the other experiment, 570 proteins were identified of which 326 (57%) were assigned as mitochondrial proteins. The fraction of peptide MS/MS spectra originating from mitochondrial proteins was 79.8%. In total, we identified 878 unique proteins of which 448 were assigned as mitochondrial (supplemental Table 1). The fraction of peptide MS/MS spectra that originated from mitochondria was 74.5%.

TABLE I
Overview of experimental strategies for mitochondrial phosphoproteomics analysis

Phosphoproteome analysis			
Amount of mitochondrial protein	Enrichment method	No. of mitochondrial phosphopeptides	No. of mitochondrial phosphoproteins
μg			
Subject 1			
200	TiO_2 microcolumn	47	28
Subject 2			
200	TiO_2 microcolumn	74	41
Subject 3			
200	TiO_2 batch	64	38
200	CPP/TiO_2 batch	56	31
200	HILIC/TiO_2 batch	77	40
Subject 4			
200	TiO_2 microcolumn	27	18
200	TiO_2 batch	29	16
200	CPP/TiO_2 batch	34	23
200	HILIC/TiO_2 batch	27	18
Subject 5			
200	TiO_2 batch	68	39
Combined		216	86

We concluded that human mitochondrial proteins were highly enriched in our protein samples. We also noted that by using the optimized mitochondrial isolation method we observed many more mitochondrial proteins than previous proteomics studies of whole muscle lysate (38) where 22% of the identified proteins were annotated as mitochondrial proteins, whereas only 8% of the peptide MS/MS spectra were assigned to mitochondrial proteins. The increase in the fraction of peptide MS/MS spectra originating from mitochondrial proteins from 8 to 75% means that ~97% of all peptide MS/MS spectra originating from non-mitochondrial proteins were removed by the isolation procedure.

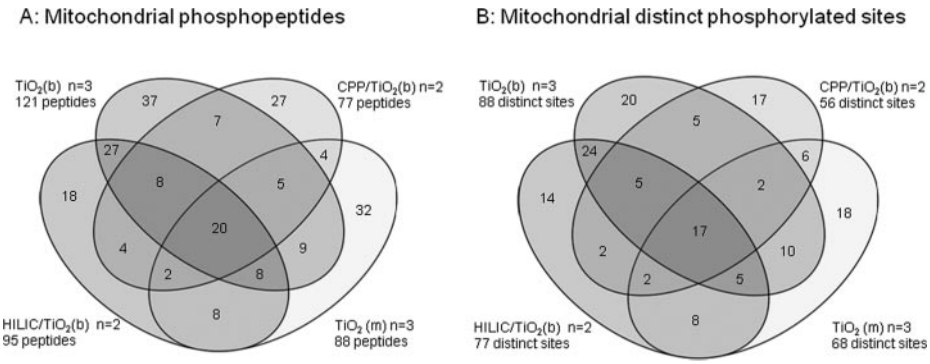

FIG. 2. **Venn diagram of identified non-redundant mitochondrial phosphopeptides (*A*) and distinct phosphorylated sites (*B*) from five subjects by four different phosphopeptide enrichment approaches.** *b*, batch mode; *m*, microcolumn mode.

Phosphopeptide Enrichment—Next, we applied several different phosphopeptide enrichment approaches with the aim to maximize the number of identified phosphorylation sites from human mitochondrial proteins. In Fig. 1 and Table I, we provide an overview of the experimental strategies used to analyze the human skeletal muscle mitochondrial phosphoproteome. Mitochondrial protein samples obtained from five subjects were digested using trypsin and enriched for phosphopeptides using TiO_2 microcolumn or TiO_2 batch-based methods, calcium phosphate precipitation followed by TiO_2 enrichment (CPP/TiO_2), or HILIC followed by TiO_2 enrichment (HILIC/TiO_2) (Fig. 1). The enriched peptide mixtures were individually analyzed by LC-MS/MS using MSA. The MS/MS spectra were searched against the IPI human database using Mascot, and the data were analyzed as described under "Experimental Procedures." Because of the limited sample amounts we could obtain from these five subjects, it was not possible to pursue a detailed and systematic comparative analysis of the different enrichment methods or to apply all methods to all subjects (Fig. 1). Nevertheless, we obtained results that gave insights into the performance and also demonstrate the complementarities of the methods as discussed below.

Fig. 2 shows the enrichment of distinct phosphopeptides assigned to mitochondrial proteins from these five subjects by four different phosphopeptide recovery methods. A total of 216 mitochondrial phosphopeptides were identified. Approaches using TiO_2 enrichment alone recovered 121 different mitochondrial phosphopeptides in batch mode and 88 phosphopeptides in the microcolumn mode, including 42 mitochondrial phosphopeptides that were identified by both protocols. HILIC and CPP were applied for prefractionation of peptides and phosphopeptides prior to TiO_2 enrichment, which greatly increased the total number of assignments, including 49 mitochondrial phosphopeptides that were not recovered by the TiO_2 batch or microcolumn enrichment protocols.

The phosphopeptide enrichment efficiency (defined here as the ratio of identified phosphopeptides to the total number of identified peptides in percent) for each enrichment technique is listed in supplemental Table 2. The efficiency was different from subject to subject and from method to method, and the highest efficiency (93.5%) was achieved by CPP/TiO_2 in Subject 4. The mitochondrial phosphoprotein enrichment efficiency, defined as the ratio of identified mitochondrial phosphoproteins to the total number of identified phosphoproteins, varied from subject to subject and from method to method and was up to 70.9% in Subject 5 by using the TiO_2 batch method. The phosphorylation profiles of mitochondria obtained from the five different human subjects are different due to *e.g.* biological variation, different enrichment methods applied, and limited replicates. A total of 49 (57%) of the mitochondrial phosphoproteins were identified by phosphopeptide MS/MS spectra in more than one subject (supplemental Table 3).

A total of 102 mitochondrial phosphopeptides were recovered by more than one enrichment approach, allowing the assignment of 86 distinct, unique phosphorylation sites (Fig. 2 and supplemental Table 3). Twenty phosphopeptides derived from 12 mitochondrial phosphoproteins were recovered by all four different approaches (Fig. 2*A*). Eleven of these abundant mitochondrial phosphoproteins and 13 of their 16 phosphorylation sites have been reported previously. Although these phosphoproteins were repeatedly detected in our study and in other studies, we still discovered one new phosphoprotein (CYC1) and three new phosphorylation sites in CYC1, CYB5R1, and HADHA, respectively. Each phosphopeptide enrichment approach also generated distinct phosphoprotein identifications (supplemental Fig. 2). In addition, the four methods recovered phosphorylated peptides that originated from different regions of a given protein. For example, this is evident for voltage-dependent anion channel (VDAC) 1, VDAC2, IMMT, and CKMT2 (Fig. 3). This illustrates the need for complementary phosphopeptide enrichment methods in phosphoproteomics. The combination of different enrichment approaches maximizes the recovery of phosphopeptides from individual proteins in a complex biological sample.

A Venn diagram was made to illustrate the overlap between the identified mitochondrial proteins and mitochondrial phos-

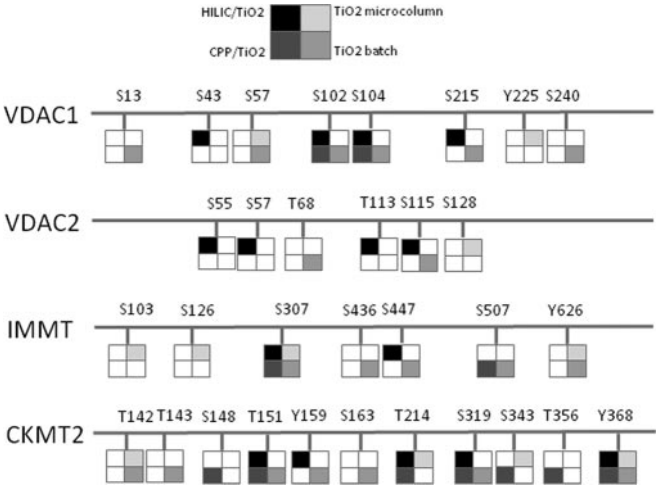

FIG. 3. **Examples of multiply phosphorylated mitochondrial proteins with indication of distinct identifications by four different phosphopeptide enrichment approaches.**

phoproteins in Subjects 1 and 2 (supplemental Fig. 3). In addition, we compared the proteome profile of functional mitochondria in human skeletal muscle reported by Lefort *et al.* (39) and the mitochondrial phosphoproteome presented here in our study (supplemental Fig. 3). Although the total number of mitochondrial proteins differs by only a few percent, ~20% of the mitochondrial phosphoproteins were not detected without the application of phosphopeptide enrichment. This further emphasizes the importance of analytical phosphoproteomics techniques.

In summary, we demonstrated that each of the different phosphopeptide enrichment methods provide distinct, overlapping, and highly informative data sets. The minor overlap between the results might be explained by the biological variation, lack of replicates due to limiting tissue sample amounts, the sequence-dependent physicochemical properties of phosphopeptides, and possibly other aspects relating to sample preparation.

Human in Vivo Skeletal Muscle Mitochondrial Phosphoproteome—We identified and characterized a total of 86 mitochondrial phosphoproteins based on 216 phosphopeptide assignments, thereby representing the largest human *in vivo* mitochondrial phosphoproteome to date.

Manual validation of phosphopeptide spectra and assignment of phosphorylation sites using strict criteria (see "Experimental Procedures") resulted in high confidence annotation of 155 phosphorylation sites in 77 mitochondrial phosphoproteins (Table II). An additional 21 ambiguous phosphorylation site assignments in 21 mitochondrial phosphoproteins are shown in Table III. Thus, a total of 176 phosphorylation sites in 86 human mitochondrial phosphoproteins are reported here. Details are provided in supplemental Table 4. Phosphoproteins are grouped according to the biological function. Among the 216 distinct mitochondrial phosphopeptides that we identified, 210 were monophosphorylated peptides, and six were doubly phosphorylated. The 155 confidently assigned phosphorylation sites include 116 phosphoserine, 23 phosphothreonine, and 16 phosphotyrosine residues (ratio of 73:14:10).

We compared our experimentally determined *in vivo* phosphorylation sites to known phosphoprotein repositories (www.phosphosite.org, www.phosida.com, www.uniprot.org, and www.phospho.elm.eu.org), and we found 134 novel phosphorylation sites (76%) and 50 novel mitochondrial phosphoproteins (58%). However, these databases and repositories are not complete because they do not list phosphoproteins detected to be phosphorylated by methods such as phosphorylation-specific antibodies and phosphoprotein-specific Pro-Q diamond staining, which provide no information on phosphorylation sites. Moreover, many studies reporting mass spectrometry-based identification of phosphorylation sites on specific proteins are often not immediately included in these databases. In fact, a number of proteomics studies have previously reported lists of mitochondrial phosphoproteins and phosphorylation sites (7, 8, 19, 29, 30, 40–47). Taking these studies into consideration, here we report 23 (27%) novel mitochondrial phosphoproteins and 29 novel phosphorylation sites in these 23 proteins. Moreover, 93 novel phosphorylation sites in 45 previously reported mitochondrial phosphoproteins were detected in our study. These novel identifications suggest a larger role of phosphorylation in regulation of mitochondrial functions than expected.

Functional Classification of Identified Mitochondrial Phosphoproteins—The 86 phosphoproteins identified were categorized into the following eight functional groups: 1) apoptosis, 2) oxidative phosphorylation (complexes I–V and electron transfer and carrier proteins), 3) tricarboxylic acid cycle (TCA) cycle, 4) fatty acid transporters and β oxidation, 5) amino acid degradation, 6) import machinery and transporters, 7) calcium homeostasis, and 8) other functions (Fig. 4).

The largest group of phosphorylation sites ($n = 31$) and phosphoproteins ($n = 23$) was identified among the respiratory complexes I–V and carrier/transfer proteins involved in oxidative phosphorylation (Fig. 5). Moreover, almost all members of the TCA cycle were found to be phosphorylated (Fig. 5). In addition, a few mitochondrial proteins involved in fatty acid transport and oxidation were also phosphorylated (Table II). Another large group of phosphorylated proteins ($n = 13$) were those involved in the mitochondrial import machinery and transport, whereas only a few phosphorylated proteins involved in apoptosis, amino acid degradation, and calcium homeostasis were identified (Table II). PDHA subunit E1, mitofilin, CKMT2, and different isoforms of VDAC and ADP/ATP translocases were phosphorylated at multiple sites, many of which appeared to be novel. Interestingly, three mitochondrial phosphoproteins (GBAS, FUNDC2, and C6orf203) with so far unknown functions were also identified. Therefore, a broad spectrum of mitochondrial proteins is a target of covalent

TABLE II

Identified distinct in vivo phosphorylation sites in isolated mitochondria from human skeletal muscle

The residue numbering is listed according to the canonical sequences by UniProt/Swiss-Prot.

UniProt accession no.	Gene name	Protein name	Phosphorylation site
Oxidative phosphorylation			
O95168	*NDUFB4*	NADH dehydrogenase 1β subcomplex subunit 4	Ser-26[a]
P17568	*NDUFB7*	NADH dehydrogenase 1β subcomplex subunit 7	Tyr-89
Q7Z4X2[a]	*NDUFB11*	NADH dehydrogenase 1β subcomplex subunit 11, variant	Tyr-133,[a] Ser-144[a]
P08574[a]	*CYC1*	Cytochrome c_1, heme protein	Ser-182[a]
P00156	*CYTB*	Cytochrome *b*	Tyr-75[a]
P31930	*UQCRC1*	Cytochrome *b*-c_1 complex subunit 1	Ser-107[a]
P22695	*UQCRC2*	Cytochrome *b*-c_1 complex subunit 2	Thr-369[a]
P47985	*UQCRFS1*	Cytochrome *b*-c_1 complex subunit Rieske	Ser-99,[a] Ser-157[a]
P99999	*CYCS*	Cytochrome *c*	Thr-29,[a] Ser-48[a]
P15954	*COX7C*	Cytochrome *c* oxidase subunit 7C	Ser-17[a]
P25705	*ATP5A1*	ATP synthase subunit α	Ser-53, Ser-65[a]
P06576	*ATP5B*	ATP synthase subunit β	Thr-213, Ser-529, Ser-415[a]
Q5VTU8	*ATP5EP2*	ATP synthase subunit ϵ-like protein	Thr-29[a]
P24539[a]	*ATP5F1*	ATP synthase subunit b	Ser-226,[a] Thr-229[a]
O75947	*ATP5H*	ATP synthase subunit d	Ser-106[a]
P18859[a]	*ATP5J*	ATP synthase-coupling factor 6	Ser-64,[a] Ser-65[a]
P48047	*ATP5O*	ATP synthase subunit O	Ser-155[a]
Q9UII2[a]	*ATPIF1*	ATPase inhibitory factor 1 isoform 3	Ser-39[a]
Tricarboxylic acid cycle			
Q99798	*ACO2*	Aconitate hydratase	Ser-669[a]
O75390	*CS*	Citrate synthase	Ser-190[a]
P10515	*DLAT*	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Ser-475[a]
P09622	*DLD*	Dihydrolipoamide dehydrogenase	Ser-297[a]
P48735	*IDH2*	Isocitrate dehydrogenase (NADP)	Ser-423[a]
P40926	*MDH2*	Malate dehydrogenase, mitochondrial	Ser-51,[a] Thr-235, Ser-246, Ser-310[a]
A5YVE9	*PDHA1*	Pyruvate dehydrogenase E1 component subunit α, somatic form	Thr-269, Ser-270, Ser-338, Ser-331
P14618	*PKM2*	Isoform M1 of pyruvate kinase isozymes M1/M2	Ser-249[a]
Q8NBX0[a]	*SCCPDH*	Probable saccharopine dehydrogenase	Ser-208[a]
Q9P2R7	*SUCLA2*	Succinyl-CoA ligase (ADP-forming) subunit β	Ser-257, Ser-259[a]
Fatty acid transporters and β oxidation			
Q92523[a]	*CPT1B*	Carnitine *O*-palmitoyltransferase 1, muscle isoform	Ser-401[a]
P43155[a]	*CRAT*	Carnitine *O*-acetyltransferase	Ser-485[a]
P42126[a]	*DCI*	3,2-*trans*-Enoyl-CoA isomerase	Ser-285[a]
P00505	*GOT2*	Aspartate aminotransferase, mitochondrial	Ser-360[a]
P40939	*HADHA*	Trifunctional enzyme subunit α	Ser-316,[a] Ser-419,[a] Ser-669[a]
Amino acid degradation			
P12694	*BCKDHA*	2-Oxoisovalerate dehydrogenase subunit α	Ser-337, Ser-347
Q96G95	*BCKDK*	Branched chain ketoacid dehydrogenase kinase, isoform CRA_a	Ser-31
Import machinery and transporters			
P35613	*BSG*	Isoform 2 of Basigin	Ser-246[a]
C9J406	*IMMT*	Putative uncharacterized protein IMMT	Ser-456[a]
Q16891	*IMMT*	Mitochondrial inner membrane protein	Ser-103,[a] Ser-126,[a] Ser-307,[a] Ser-436,[a] Ser-447,[a] Ser-507, Tyr-626[a]
Q9Y512	*SAMM50*	Sorting and assembly machinery component 50 homolog	Ser-270
Q02978	*SLC25A11*	Mitochondrial 2-oxoglutarate/malate carrier protein	Ser-6,[a] Tyr-102, Tyr-202, Ser-203[a]

TABLE II—*continued*

UniProt accession no.	Gene name	Protein name	Phosphorylation site
O75746[a]	SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	Ser-101,[a] Ser-662[a]
O43772[a]	*SLC25A20*	Mitochondrial carnitine/acylcarnitine carrier protein	Ser-205[a]
Q59EP7	*SLC25A4*	Solute carrier family 25 member 4, variant	Ser-200[a]
P12235	*SLC25A4*	ADP/ATP translocase 1	Ser-7, Ser-22, Tyr-191
P05141	*SLC25A5*	ADP/ATP translocase 2	Thr-84
P12236	*SLC25A6*	ADP/ATP translocase 3	Tyr-112,[a] Ser-119,[a] Tyr-195
O60220	*TIMM8A*	Mitochondrial import inner membrane translocase subunit Tim8 A	Ser-57[a]
P21796	*VDAC1*	Voltage-dependent anion-selective channel protein 1	Ser-13, Ser-43,[a] Ser-57, Ser-102,[a] Ser-104, Ser-215, Tyr-225,[a] Ser-240
P45880	*VDAC2*	Voltage-dependent anion-selective channel protein 2	Ser-55,[a] Ser-57,[a] Thr-68,[a] Thr-113, Ser-115, Ser-128[a]
Q9Y277	*VDAC3*	Voltage-dependent anion-selective channel protein 3	Thr-4, Thr-6,[a] Ser-45,[a] Tyr-49, Thr-71[a]
Calcium homeostasis			
B1AKZ2	*CASQ1*	Calsequestrin	Ser-191[a]
P26678	*PLN*	Cardiac phospholamban	Ser-16, Thr-17
Apoptosis			
O95831	*AIFM1*	Apoptosis-inducing factor 1	Ser-268, Ser-279,[a] Ser-371, Ser-376[a]
P17252	*PRKCA*	Protein kinase C α type	Ser-226
Others			
O00116	*AGPS*	Alkyldihydroxyacetonephosphate synthase, peroxisomal	Ser-65
Q9BUR5[a]	*APOO*	Apolipoprotein O	Ser-44[a]
Q9GZT3	*C14orf156*	SRA stem-loop-interacting RNA-binding protein	Ser-102
Q5TGZ0[a]	*C1orf151*	UPF0327 protein C1orf151	Ser-4[a]
Q9NX63	*CHCHD3*	Coiled coil-helix-coiled coil-helix domain-containing protein 3	Ser-29,[a] Ser-46, Tyr-49, Ser-50
Q9NZ45[a]	*CISD1*	CDGSH iron-sulfur domain-containing protein 1 (MitoNEET)	Ser-2,[a] Ser-7[a]
P17540	*CKMT2*	Creatine kinase S-type, mitochondrial	Thr-142,[a] Thr-143,[a] Ser-148,[a] Thr-151,[a] Tyr-159,[a] Ser-163,[a] Thr-214,[a] Ser-319, Ser-343, Thr-356,[a] Tyr-368
O75208	*COQ9*	Ubiquinone biosynthesis protein COQ9	Thr-178[a]
Q9UHQ9	*CYB5R1*	NADH-cytochrome b_5 reductase 1	Thr-56,[a] Tyr-84, Ser-150[a]
P00387[a]	*CYB5R3*	NADH-cytochrome b_5 reductase 3	Ser-146[a]
Q9BWH2	*FUNDC2*	FUN14 domain-containing protein 2	Ser-151, Ser-167[a]
O75323[a]	*GBAS*	Protein NipSnap homolog 2	Ser-182[a]
P19367	*HK1*	Hexokinase-1	Ser-337,[a] Ser-340[a]
P08238	*HSP90AB1*	Heat-shock protein HSP90-β	Ser-255
P83111[a]	*LACTB*	Serine β-lactamase-like protein LACTB	Ser-532[a]
P21397	*MAOA*	Amine oxidase (flavin-containing) A	Tyr-53[a]
P27338[a]	*MAOB*	Amine oxidase (flavin-containing) B	Ser-374[a]
P54296	*MYOM2*	Myomesin-2	Ser-59,[a] Ser-76, Ser-192,[a] Thr-600,[a] Ser-737,[a] Ser-946,[a] Ser-951[a]
P35232	*PHB*	Prohibitin	Ser-101,[a] Thr-108[a]
Q99623[a]	*PHB2*	Prohibitin-2	Ser-91,[a] Ser-151,[a] Thr-155[a]
Q9H7Z7[a]	*PTGES2*	Prostaglandin E synthase 2	Ser-97[a]
P49411	*TUFM*	Elongation factor Tu	Ser-312[a]

[a] Novel phosphoproteins/phosphorylation site.

TABLE III

Identified ambiguous phosphorylation sites in isolated mitochondria from human skeletal muscle

The residue numbering is listed according to the canonical sequences by UniProt/Swiss-Prot. /, ambiguous site.

UniProt accession number	Gene name	Protein name	Phosphorylation site
Oxidative phosphorylation			
O95167	*NDUFA3*	NADH dehydrogenase 1α subcomplex subunit 3	Tyr-41/Ser-42[a]
P56181	*NDUFV3*	NADH-ubiquinone oxidoreductase flavoprotein 3	Ser-59/Ser-60/Ser-62/Ser-64
P13804	*ETFA*	Electron transfer flavoprotein subunit α	Ser-189/Ser-190[a]
P07919	*UQCRH*	Cytochrome b-c_1 complex subunit 6	Thr-63/Thr-68[a]
P13073	*COX4I1*	Cytochrome c oxidase subunit 4 isoform 1	Ser-71/Ser-72[a]
Tricarboxylic acid cycle			
Q99798	*ACO2*	Aconitate hydratase	Ser-669/Ser-670[a]
Q86SW4[a]	*DLST*	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	Ser-64/Thr-66[a]
P07954	*FH*	Fumarate hydratase	Ser-65/Ser-66[a]
P48735	*IDH2*	Isocitrate dehydrogenase (NADP)	Ser-300/Ser-301[a]
Amino acid degradation			
P51649[a]	*ALDH5A1*	Succinate-semialdehyde dehydrogenase	Ser-285/Thr-286/Thr-287[a]
P12694	*BCKDHA*	2-Oxoisovalerate dehydrogenase subunit α	Tyr-345/Ser-347
Q96G95	*BCKDK*	Branched chain ketoacid dehydrogenase kinase, isoform CRA_a	Ser-31/Thr-32/Ser-33
Import machinery and transporters			
Q16891	*IMMT*	Mitochondrial inner membrane protein	Thr-582/Ser-583/Ser-584[a]
P21796	*VDAC1*	Voltage-dependent anion-selective channel protein 1	Ser-240/Ser-241
P45880	*VDAC2*	Voltage-dependent anion-selective channel protein 2	Ser-251/Ser-252[a]
Calcium homeostasis			
B1AKZ2[a]	*CASQ1*	Calsequestrin	Thr-213/Ser-216[a]
Others			
Q9P0P8	*C6orf203*	Uncharacterized protein C6orf203	Ser-106/Ser-110
P17540	*CKMT2*	Creatine kinase S-type, mitochondrial	Tyr-207/Tyr-208[a]
Q9H3Z4	*DNAJC5*	DnaJ homolog subfamily C member 5	Ser-8/Ser-10
P19367	*HK1*	Hexokinase-1	Ser-336/Ser-337[a]
P35232	*PHB*	Prohibitin	Thr-23/Thr-24[a]

[a] Novel phosphoproteins/phosphorylation site.

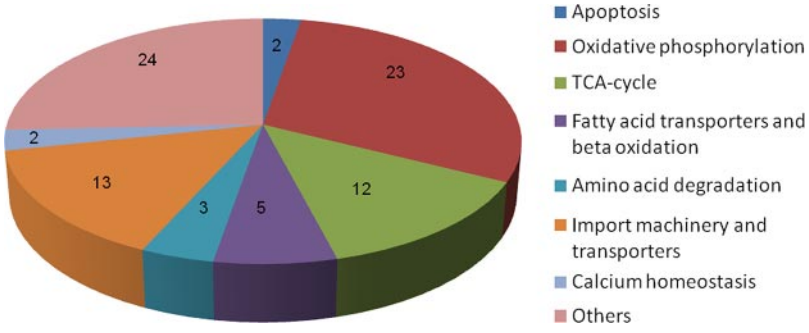

FIG. 4. **Functional classification of all identified human mitochondrial phosphoproteins.**

modification, suggesting that many mitochondrial functions are regulated by reversible protein phosphorylation.

Potential Kinases for Identified Phosphorylation Sites—Our data set provides a source of phosphopeptide sequences for exploring the network between different kinase families and their substrates. We used NetworKIN (48) to generate a list of predicted potential kinases for each of the 155 distinct mitochondrial phosphorylation sites (supplemental Table 5). Prediction was based on comparison of the phosphorylation sites assigned in our study with reported consensus sequences for different protein kinases. The number of distinct phosphorylation sites for each kinase family (Fig. 6) suggests that the greatest proportion (78%) of the identified distinct phosphorylation sites is potential substrates of CKII, PKA, PKC, or DNAPK. In a phosphoproteomics study of human skeletal muscle (34), the kinase prediction of identified phosphopro-

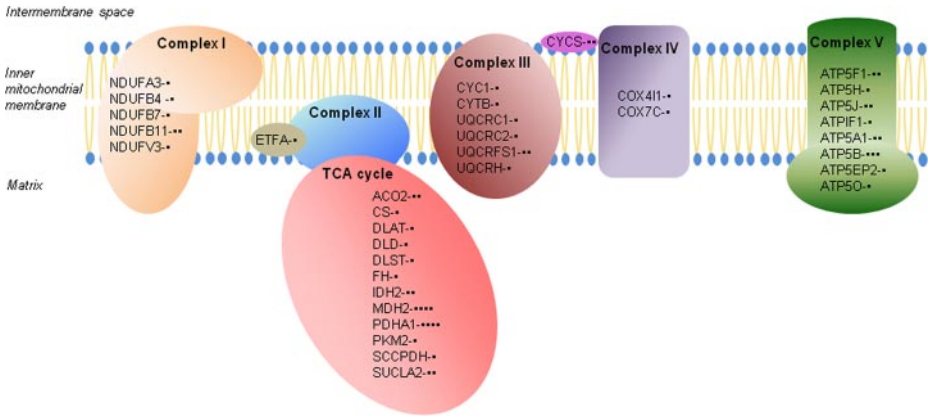

FIG. 5. **Schematic of mitochondrial respiration chain and TCA cycle and identified phosphoproteins involved in these two processes.**"●" indicates the number of site-specific phosphorylations.

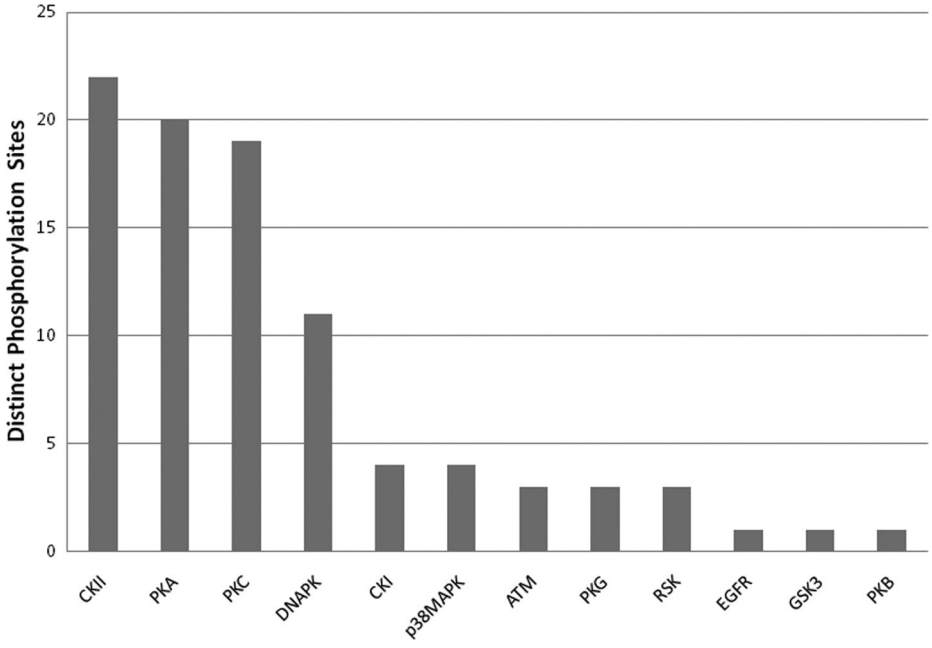

FIG. 6. **Prediction of kinase family for all identified distinct phosphorylation sites by NetworKIN.** *CKI*, casein kinase I; *EGFR*, EGF receptor.

teins suggested a major role for the CKII, PKA, and PKC kinase families but not for DNAPK.

DISCUSSION

Here we present the first systematic study of the *in vivo* mitochondrial phosphoproteome in human skeletal muscle. Combining different phosphopeptide enrichment approaches with high performance LC-MS/MS, 176 phosphorylation sites in 86 mitochondrial phosphoproteins were identified. A previous study of human skeletal muscle by Højlund *et al.* (34) reported the identification of 367 phosphorylation sites in 144 phosphoproteins using the combination of strong cation exchange and TiO_2. Of these, 22% were assigned as mitochondrial phosphoproteins. Our results demonstrate that the identification of phosphorylation sites in mitochondrial proteins by LC-MS/MS is highly improved by fractionation of mitochondria from human skeletal muscle tissue prior to phosphopeptide enrichment. The phosphopeptide enrichment efficiency was 55–93% by using TiO_2 microcolumn, TiO_2 batch, HILIC/TiO_2, or CPP/TiO_2. Thus, recovery of phosphopeptides using different enrichment techniques turned out to be very effective (Table I).

Our data are in agreement with a number of recent studies, which have indirectly shown a number of mitochondrial proteins to be phosphorylated in other tissues and species (41, 43). Among 45 of these previously reported mitochondrial phosphoproteins, we found several novel phosphorylation sites. Moreover, many of the phosphorylation sites identified in this study have been reported before in various other studies of mitochondria from different species, tissues, and cell types. The largest overlap was observed by comparison of our data with the recent quantitative study of isolated mitochondria from pig heart muscle by Boja *et al.* (29). Thus, 42% (22) of the 52 known phosphorylation sites identified in our study were also reported by Boja *et al.* (29).

Of all the identified mitochondrial phosphoproteins, MitoNEET (CISD1; CDGSH iron-sulfur domain-containing protein 1), which is a uniquely folded 2Fe-2S outer mitochondrial membrane protein, is of great interest. MitoNEET is emerging as a new potential target for type 2 diabetes treatment using pioglitazone, which is a member of the thiazolidinedione family (49–52). The stability of the 2Fe-2S cluster is increased by binding to pioglitazone. MitoNEET is believed to play an important role in regulating maximal capacity for electron transport and oxidative phosphorylation. However, so far, no phosphorylation sites of MitoNEET have been described. In our study, two distinct phosphorylation sites, Ser-2 and Ser-7, were identified in the peptide SLTSSSSVR, which is a part of the putative N-terminal transmembrane helix (residues 1–31). This N-terminal sequence of MitoNEET is not well studied but seems to be the membrane-spanning part, targeting MitoNEET to the outer mitochondrial membrane (50). Thus, phosphorylation of this signal sequence may play an important role in the targeting or binding of MitoNEET to the mitochondrial membrane. The discovery of these novel phosphorylation sites may contribute to reveal the function and biological activity of human MitoNEET and hence its value as a target for treatment of type 2 diabetes.

The kinase family prediction provided by NetworKIN does not include all kinase families. However, the prediction produced quite similar results in the basal state as in a phosphoproteomics analysis of whole muscle (34), which also showed a major role for PKA, CKII, and PKC. Interestingly, our results imply a greater role for DNAPK and perhaps a relatively reduced role for GSK3, RSK, and CDK5 in mitochondria. Indeed, carnitine palmitoyltransferase 1 and glycerophosphate acyltransferase in mitochondria were previously shown to be phosphorylated by CKII (53, 54). It was reported that DNAPK may phosphorylate mitochondrial heat-shock proteins in treatment with anticancer drugs (55). However, the role of PKC and PKA in the phosphorylation of mitochondrial proteins is better documented. Different isoforms of PKC are known to translocate into mitochondria and localize to the inner membrane and cristae, playing a direct role in regulating mitochondrial functions (10). In fact, we identified a PKC isoform, PKCα, in our preparation. PKCα has been reported to play a functional role in reducing apoptosis by phosphorylating mitochondrial Bcl-2 (56). Another isoform, PKCε, is also known to mediate its antiapoptotic effect via mitochondria (57). Moreover, PKCε has been reported to promote cardioprotection by phosphorylating VDAC (58). Here in our study, 19 distinct phosphorylation sites were identified in VDAC1, VDAC2, and VDAC3, many of which were not reported before. Four phosphorylation sites (Ser-215 in VDAC1, Ser-128 in VDAC2, Ser-45 and Thr-71 on VDAC3) were predicted as targets of PKC by NetworKIN. These novel identifications suggest a potential great impact of PKC phosphorylation on VDAC isoforms through which mitochondrial processes are regulated. Potential PKA substrates are also well represented among the mitochondrial phosphoproteins identified in the present study. PKA is responsive to elevated cAMP levels and mediates signaling cascades. PKA activity has been reported in human mitochondria (7, 8, 10). Translocated to the inner membrane and matrix of mitochondria, PKA has been shown to have positive effects on most major mitochondrial functions, enhancing cellular respiration rate, inhibiting apoptosis, and regulating protein expression and biogenesis (10). Although these previous studies support our findings, the role of these kinases on the specific phosphorylation sites needs to be characterized in future studies.

The relative abundance of phosphotyrosine residues was recently shown to be 2–5-fold higher in skeletal muscle than in other human tissue or cell types, suggesting a potential enhanced role for tyrosine phosphorylation in this mitochondrion-rich tissue (34). Consistent with this finding, here we report that the extent of phosphotyrosine residues in isolated muscle mitochondria is up to 12-fold higher than in other human tissue and cell types (59–62). This high ratio was unexpected because it was observed in a non-stimulated condition in which tyrosine phosphorylation is believed to be at low levels (40). The phosphotyrosine residues identified in the present study were located in subunits of complex I or complex III (electron transport chain) and outer or inner membrane solute carriers and channel proteins, but other studies have shown that tyrosine phosphorylated proteins are distributed to almost all mitochondrial compartments (8, 40, 42). Accordingly, a number of tyrosine-specific kinases and phosphatases have been localized to mitochondria, and a role for tyrosine phosphorylation in the regulation of mitochondrial function has gained increasing attention (7, 8, 40). There is evidence that tyrosine phosphorylation of specific subunits of cytochrome *c* oxidase (complex IV) is capable of both inhibiting (63) and activating (64) complex IV activity and hence ATP synthesis, but most of the tyrosine phosphorylated sites have been identified in global phosphoproteomics surveys (42, 62) and therefore need to be studied further. Taken together, our data lend support to the hypothesis that tyrosine phosphorylation could represent a new frontier in mitochondrial signaling (42). It is expected that the use of muscle mitochondria isolated after *in vivo* exposure to stimuli such as contraction and insulin in combination with phosphotyrosine enrichment prior to MS/MS would lead to the identification of a higher number of tyrosine phosphorylated residues and that this will help to reveal the role of these PTMs in human muscle mitochondria in both healthy and diseased states such as insulin resistance.

The identified mitochondrial phosphoproteins are involved in a broad range of biological processes crucial for mitochondrial and muscle metabolism, including oxidative phosphorylation, TCA cycle, fatty acid metabolism, amino acid degradation, cell survival, calcium homeostasis, and outer and inner membrane solute carriers and channel proteins. Even proteins with so far unknown functions were

identified. Our results are in accordance with a number of recent reports indicating that phosphorylation of mitochondrial proteins may play a greater role than hitherto expected. A high number of subunits involved in mitochondrial oxidative phosphorylation are phosphorylated even in the basal state (Fig. 5). Moreover, almost all enzymes in the TCA cycle and a number of enzymes involved in fatty acid metabolism are phosphorylated. These mitochondrial processes play a crucial role for the balance between glucose and lipid metabolism in human skeletal muscle. Our results suggest that reversible phosphorylation of mitochondrial proteins may play a role in the association between mitochondrial dysfunction and insulin resistance in obesity and type 2 diabetes (32) and other muscle disorders characterized by abnormal mitochondrial content and function. Therefore, these data provide potential targets for investigation of functional implications under different conditions and for treatment in muscular diseases.

The present study is a "discovery mode" proteomics study, and although a high number of phosphorylation sites were identified, more sites could probably be identified by hypothesis-driven targeted phosphoproteomics analysis of each potential phosphorylation site in these mitochondrial phosphoproteins. We detected only one (at Thr-213) of the seven phosphorylation sites identified in a recent study of immunopurified ATP synthase β by targeted phosphoproteomics (65). However, two additional phosphorylation sites at Ser-415 and Ser-529 in ATP synthase β were identified in our study. Our data represent a comprehensive first attempt for the characterization of the *in vivo* mitochondrial phosphoproteome in human skeletal muscle and provide a long list of mitochondrial proteins for large scale targeted phosphoproteomics analysis. This list includes so far only mitochondrial proteins phosphorylated to a detectable extent in the resting, basal state. The number of mitochondrial phosphoproteins and phosphorylation sites may increase significantly in human skeletal muscle in response to different stimuli, including insulin, *e.g.* via translocation of signaling enzymes from cytosol to mitochondria, which has been shown for PKB/Akt (66, 67), or exercise/contraction, *e.g.* mediated by changes in calcium concentration (41). Also the application of more efficient phosphoenrichment procedures, in particular when using small human samples, may significantly increase the number of phosphorylation sites identified.

In summary, this study presents the *in vivo* phosphoproteome of isolated mitochondria from small human skeletal muscle biopsies. These results provide a rich source for investigating the role of reversible phosphorylation in mitochondrial functions in healthy and pathological conditions and also for ongoing targeted clinical phosphoproteome studies.

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S This article contains supplemental Figs. 1–4 and Tables 1–5.

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