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Mitochondrial phosphoproteomics of mammalian tissues

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ABSTRACT

Mitochondria are essential for several biological processes including energy metabolism and cell survival. Accordingly, impaired mitochondrial function is involved in a wide range of human pathologies including diabetes, cancer, cardiovascular, and neurodegenerative diseases. Within the past decade a growing body of evidence indicates that reversible phosphorylation plays an important role in the regulation of a variety of mitochondrial processes as well as tissue-specific mitochondrial functions in mammals. The rapidly increasing number of mitochondrial phosphorylation sites and phosphoproteins identified is largely ascribed to recent advances in phosphoproteomic technologies such as fractionation, phosphopeptide enrichment, and high-sensitivity mass spectrometry. However, the functional importance and the specific kinases and phosphatases involved have yet to be determined for the majority of these mitochondrial phosphorylation sites. This review summarizes the progress in establishing the mammalian mitochondrial phosphoproteome and the technical challenges encountered while characterizing it, with a particular focus on large-scale phosphoproteomic studies of mitochondria from human skeletal muscle.

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1. Introduction

Protein phosphorylation is one of the most abundant post-translational modifications (PTMs) and is known to be involved in a variety of cellular functions, including cell signaling, metabolism, protein degradation, and cell differentiation (Hunter, 2000; Graves and Krebs, 1999). In eukaryotes, protein phosphorylation occurs at serine, threonine, and tyrosine residues and is regulated by the dynamic interplay between protein kinases and phosphatases. Protein phosphorylation is a transient reversible modification that may take place on a very short timescale, thus resulting in a rapid change in protein structure, interaction, function, or localization. As such, protein phosphorylation is a key regulatory mechanism that has a great impact on most essential cellular processes.

Mitochondria are essential in energy metabolism and for cellular survival and play a central role in numerous metabolic processes such as oxidative phosphorylation (OXPHOS), the tricarboxylic acid (TCA) cycle, β-oxidation, amino acid metabolism, apoptosis, and calcium homeostasis (Johannsen and Ravussin, 2009; Nunnari and Suomalainen, 2012; Raimundo, 2014). Consequently, impaired mitochondrial function is associated with a vast array of human disorders, including diabetes, cancer, cardiovascular disease, and neurodegenerative diseases (Johannsen and Ravussin, 2009; Nunnari and Suomalainen, 2012; Raimundo, 2014; Pagel-Langenickel et al., 2010; Hojlund et al., 2008a). The first evidence of reversible protein phosphorylation in mitochondria was reported when the E1 subunits of the pyruvate dehydrogenase and the branched-chain alpha-ketoacidose dehydrogenase complexes were identified as mitochondrial phosphoproteins (Linn et al., 1969; Wieland and Jagow-Westermann, 1969; Wieland and Siess, 1970; Hughes and Halestrap, 1981; Harris et al., 1986; Patel and Olson, 1982). Subsequently, growing evidence suggests that multiple mitochondrial proteins involved in key regulatory processes are phosphorylated in different mammalian tissues (Pagliarini and Dixon, 2006; Covian and Balaban, 2012; Bak et al., 2013; Zhao et al., 2011; Zhao et al., 2014; Deng et al., 2010; Deng et al., 2011; Feng et al., 2008; Grimsrud et al., 2012; Hopper et al., 2006; Lee et al., 2007). At least in the last decade, the increasing number of mitochondrial phosphoproteins being identified may be largely ascribed to the recent advances in mass spectrometry.

Mass spectrometry is a potent tool in the identification and characterization of PTMs (Jensen, 2006). The improved technical and analytical capabilities of mass spectrometry have contributed greatly to the identification of low stoichiometry PTMs in complex samples. With

Abbreviations: CID, Collision-induced dissociation; CPP, Calcium phosphate precipitation; ETD, Electron transfer dissociation; HILIC, Hydrophilic interaction chromatography; IMAC, Immobilized metal affinity chromatography; iTRAQ, Isobaric tags for relative and absolute quantification; MALDI, Matrix-assisted laser desorption-ionization; MS/MS, Tandem mass spectrometry; OXPHOS, Oxidative phosphorylation; PTMs, Post-translational modifications; SCX, Strong cation exchange chromatography; SIMAC, Sequential IMAC; TCA, Tricarboxylic acid; TiO_2, Titanium dioxide; T2D, Type 2 diabetes; 2-DE, Two-dimensional gel electrophoresis.

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the present review, we aim to address the mitochondrial phosphoproteome in mammalian tissues with a particular focus on large-scale phosphoproteomic studies in human skeletal muscle.

1.1. Strategies for phosphoproteomics

PTMs are covalent modifications that may change the activity, localization, turnover, or interaction partners of the protein being modified. Traditionally, a protein modification has been associated with one specific regulatory event but the expanding number of identified PTMs suggests that they also should be looked at as a whole as they function together to fine-tune protein interactions as well as protein activity and stability (Jensen, 2006). Large-scale phosphoproteomic studies are complicated by the complexity of protein phosphorylation and the number of phosphorylation sites. Over two-thirds of the ~23,000 proteins encoded by the human genome are phosphorylated and the number of phosphorylation sites in human proteins is estimated to exceed 650,000 (Zhang and Pelech, 2012). Whether these phosphorylation sites have a physiological relevance should obviously be questioned, but >100,000 sites have already been experimentally confirmed in humans and/or related mammals (Zhang and Pelech, 2012). It is therefore an overwhelming and huge task to interpret the dynamics of the phosphoproteome.

Protein phosphorylation is by far the most studied PTM based on the number of publications (Zhang and Pelech, 2012). The development of techniques such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry have shifted phosphoproteomic research from a single-protein approach into a global approach with the aim of identifying and quantifying the largest possible number of phosphorylation sites in whole-cell lysates or subcellular fractions. Compared with studies targeted towards the phosphorylation status of a particular protein of interest, the global approach represents an unbiased way of observing cellular phosphorylation events in the absence of á priori knowledge.

2-DE analysis has commonly been used in global phosphoproteomic studies to separate highly complex protein samples according to their pI and molecular weight. Among the advantages of 2-DE is that the removal or addition of a functional group, e.g. a phosphate-moiety, alters the pI, thereby leading to a shift in protein localization on the gel (Thingholm et al., 2009a; Reinders and Sickmann, 2005). Additionally, the fact that the protein digest of an excised gel spot has a relatively low complexity has improved the detection of phosphopeptides by mass spectrometry analysis (Sun et al., 2012). On the negative side, 2-DE separation is limited by its inherent resolving power. Moreover, 2-DE is generally biased against hydrophobic proteins, such as membrane and nuclear proteins (Santoni et al., 2000). Phosphorylated proteins that are separated by 2-DE may be visualized using various different staining protocols (Steinberg, 2009). Autoradiography using ^{32}P labelling of phosphate groups and their subsequent detection by autoradiography is considered a sensitive and unbiased method for detection of phosphorylated proteins on a 2-D gel (Thingholm et al., 2009a; Reinders and Sickmann, 2005; Sun et al., 2012; Pocsfalvi, 2009) but also fluorescent stains, such as Pro-Q Diamond, are commonly used for detection of phosphoproteins (Steinberg, 2009). In fact, Hopper et al. (Hopper et al., 2006) have shown that Pro-Q Diamond staining is comparable to ^{32}P labelling in terms of sensitivity. Accordingly, both methods have been used in several studies to investigate the mitochondrial phosphoproteome of mammalian heart and liver (Hopper et al., 2006; Lee et al., 2007; Aponte et al., 2009a; Aponte et al., 2009b; Clarke et al., 2008; Schulenberg et al., 2003). An advantage of fluorescent stains over autoradiography is the fact that they can detect phosphoproteins in all cellular states and sample types, as they do not require protein turnover for the incorporation of radioactive phosphate (Thingholm et al., 2009a; Sun et al., 2012). The 2-DE and labelling-dependent strategies have contributed to the characterization of e.g. the mitochondrial phosphoproteome. However, due to the recent advances in mass spectrometry instrumentation, including improved sensitivity, resolution, and a higher sequencing speed (Thingholm et al., 2009a; Sun et al., 2012; Pocsfalvi, 2009) as well as the optimized protocols for selective enrichment of phosphopeptides (Sun et al., 2012; Beltran and Cutillas, 2012), mass spectrometry-based phosphoproteomics has emerged as the method of choice for studying protein phosphorylation. Shot-gun mass spectrometry makes it possible to characterize the phosphoproteome in large-scale, non-targeted, discovery-mode phosphoproteomic studies without focusing on a specific protein or a specific phosphorylation site. Targeted proteomics, such as selected/multiple reaction monitoring (SRM/MRM), on the other hand, offers the possibility of looking specifically at multiple proteins of interest with a higher sensitivity and throughput than shot-gun proteomics (Boja and Rodriguez, 2012).

1.2. Phosphopeptide enrichment

It is a difficult task to detect protein phosphorylation since these PTMs are usually transient and low abundant. The large dynamic range of protein abundances in whole-cell lysates, in particular in mammalian skeletal muscle, is a challenge when characterizing the proteome and its PTMs in a particular tissue or its organelles. The development of techniques for selective enrichment of phosphopeptides from complex samples and novel mass spectrometry applications and bioinformatic tools have together with subcellular fractionation greatly expanded the coverage of the mitochondrial phosphoproteome (Deng et al., 2011; Boja et al., 2009; Gaucher et al., 2004). The low stoichiometry of most phosphoproteins (ratio of phosphorylated to non-phosphorylated counterparts) represents a huge analytical challenge in proteomic research since complex biological samples consist of thousands of proteins differing in abundance over four or more orders of magnitude (Thingholm et al., 2009a). Enrichment of phosphopeptides prior to mass spectrometry-based analysis will therefore greatly improve the outcome. The necessity of phosphopeptide enrichment is underlined by the fact that only few phosphorylation sites were identified by discovery-mode mass spectrometry analysis in human skeletal muscle or isolated muscle mitochondria when no attempts were made to enrich phosphopeptides (Lefort et al., 2009; Hojlund et al., 2008b; Yi et al., 2008).

There are various approaches available for phosphopeptide enrichment, including strong cation exchange chromatography (SCX), immobilized metal affinity chromatography (IMAC), metal oxide affinity chromatography using titanium dioxide (TiO_2), calcium phosphate precipitation (CPP), and immunoprecipitation with anti-phosphotyrosine or anti-phosphoserine/threonine antibodies (Thingholm et al., 2009a; Reinders and Sickmann, 2005; Sun et al., 2012; Schmelzle and White, 2006). The present review will only briefly comment on the technical aspects of phosphoproteomic studies and refers to the many excellent reviews of this topic for additional information (Thingholm et al., 2009a; Reinders and Sickmann, 2005; Sun et al., 2012).

Traditionally, antibody-based enrichment through immunoprecipitation of phosphoproteins using phospho-tyrosine or phospho-serine/threonine-specific antibodies has been utilized to minimize sample complexity. Generally, this approach is mostly targeted towards phosphorylated tyrosine residues due to a better quality of antibodies (Thingholm et al., 2009a; Reinders and Sickmann, 2005). For investigation of phosphorylation sites in a protein of interest, immunoprecipitation of this specific protein using protein-specific antibodies prior to non-targeted or targeted MS/MS analysis is an often-applied approach (Hojlund et al., 2010; Langlais et al., 2010; Langlais et al., 2011). However, it is important to keep in mind that the presence of PTMs may mask or alter the protein epitope thus making the yield of immunoprecipitation lower (Jensen, 2006).

Phosphopeptides are commonly enriched by IMAC, which is an affinity-based technique based on the binding of phosphate-groups to immobilized metal ions (Fe^{3+}, AL^{3+}, Ga^{3+}, or Co^{3+}). The metal ions

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