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Identification of new tyrosine phosphorylated proteins in rat brain mitochondria

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Abstract **Reversible protein-phosphorylation is emerging as a key player in the regulation of mitochondrial functions. In particular tyrosine phosphorylation represents a promising field to highlight new mechanisms of bioenergetic regulation.**

Utilizing immunoaffinity enrichment of phosphotyrosine-containing peptides coupled to mass spectrometric analysis we detected new tyrosine phosphorylated proteins in rat brain mitochondria after peroxovanadate treatment. By bioinformatic predictions we provide suggestions about the potential role of tyrosine phosphorylation in mitochondrial physiology. Our results indicate a primary role of tyrosine phosphorylation in regulating energy production at the mitochondrial level. Moreover, tyrosine phosphorylation might regulate the mitochondrial membrane permeability targeting protein complexes containing ADP/ATP translocase, VDAC, creatine kinase and hexokinase.

Keywords: Mitochondria; Tyrosine phosphorylated protein; Rat brain; Energy production; Membrane permeabilization

1. Introduction

Reversible protein-phosphorylation is emerging as a key player in the regulation of mitochondrial functions. First drafts of a mitochondrial Ser/Thr phosphoproteome have been recently published [1,2], highlighting a role of reversible phosphorylation in different mitochondrial processes [3] and several Ser/Thr kinases and phosphatases have been described in mitochondrial compartments [3]. Tyrosine phosphorylation is also emerging as a central mechanism for regulating mitochondrial functions, and the involvement of tyrosine phosphorylation in mitochondrial signaling is discussed in depth in a recent review [4]. To date, only tyrosine kinases belonging to the Src family have been described in mitochondrial compartments such as Lyn, c-Src, Fyn and Fgr [5–8]. In this context, at least two different anchoring proteins, AKAP121 [7] and Dok-4 [8], regulate the association of c-Src with mitochondria. The reversibility of phosphorylation is guaranteed by the presence of tyrosine phosphatases: a pool of shp-2 has been described in mitochondrial compartments [9] and more important, the PTPM-1 (protein tyrosine phosphatase localized to the mitochondrion 1), has been discovered as the first tyrosine phosphatase with an almost exclusive mitochondrial localization [10]. Tyrosine phosphorylation seems to have a primary role in regulating electron-transport chain (ETC) activity. Cytochrome *c* oxidase (COX), the terminal respiratory complex of ETC, has been identified as a substrate for the tyrosine kinase c-Src [6]. C-Src phosphorylates COX subunit II in vitro and in osteoclasts at so far unidentified site/s; it thereby activates COX and, in turn, increases the efficiency of the mitochondrial ETC [6]. Tyrosine phosphorylation of COX subunit I on Y304 by an unidentified kinase and upon activation of the cAMP-dependent pathway, leads to opposing effects on the activity of the enzyme [11]. Increased targeting of c-Src to mitochondria by AKAP121 enhances mitochondrial membrane potential and ATP oxidative synthesis in a c-Src dependent manner [7]. Overexpression of Dok-4 is associated with a decrease of the expression of Complex I, strictly dependent to the activity of the c-Src kinase activity, modulating also the subsequent production of reactive oxygen species [8].

A reduction in mitochondrial tyrosine phosphoprotein profile by the knock-down of PTPM-1 in pancreatic insulinoma cell line INS-1 markedly enhances ATP production at the mitochondrial level [10].

Recently, new tyrosine phosphorylated mitochondrial proteins and their phosphorylation sites have been identified by mass proteomic approaches (see below), but the definition of a complete mitochondrial tyrosine phosphoproteome is only at the very beginning. Utilizing the KESTREL approach, we have previously identified the flavoprotein of succinate dehydrogenase and aconitase as in vitro mitochondrial substrates of Fgr by identification of the respective phosphorylation sites [12].

Here we identify new tyrosine phosphorylated proteins in rat brain mitochondria after peroxovanadate treatment by immunoaffinity enrichment of phosphotyrosine-containing peptides coupled to mass spectrometry analysis. We also provide information regarding the potential role of tyrosine phosphoryla-

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Abbreviations: ANT, ADP/ATP translocase; COX, cytochrome *c* oxidase; mtCK, mitochondrial creatine kinase; ETC, electron-transport chain; HK-I, Type I hexokinase; MPT, mitochondrial permeability transition; PTPM-1, protein tyrosine phosphatase localized to the mitochondrion 1; RBM, rat brain mitochondria; VDAC, voltage-dependent anion channel

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tion of these proteins in mitochondrial physiology by means of bioinformatic tools.

2. Materials and methods

2.1. Isolation and purification of rat brain mitochondria

Rat brain mitochondria (RBM) were isolated by differential centrifugation and purified by the Ficoll gradient method as described in Supplementary methods online.

2.2. RBM incubation

RBM (20 mg) were resuspended in 1 ml buffer containing 200 mM sucrose, 50 mM HEPES, 0.5 mM EDTA, pH 7.4. At t_0 1 mM peroxovanadate, 1 mM $MnCl_2$, 1 mM ATP and 1 μg/ml olygomycin was added and the sample was incubated for 20 min at 30 °C. The reaction was stopped by centrifugation for 10 min at $12000 \times g$. The assessment of mitochondrial integrity during peroxovanadate treatment was tested in a control experiment without olygomycin as described in Supplementary methods online.

2.3. Additional methods

Methods for mitochondrial lysis, digestion, phosphopeptide isolation and purification as well as mass spectrometric analysis are available in Supplementary methods online.

3. Results and discussion

In order to identify novel tyrosine phosphorylated mitochondrial proteins we utilized a targeted proteomic approach combined with the immunoaffinity enrichment of phosphotyrosine-containing peptides. Mitochondria were isolated from rat brain because a previous tyrosine kinase assay on the generic tyrosine kinase substrate PolyGlu $Tyr_{(4:1)}$, has demonstrated a much higher tyrosine kinase activity in brain mitochondria than in mitochondria purified from rat liver and heart [12], thus resulting in a higher probability to identify tyrosine phosphorylated mitochondrial proteins. Isolated mitochondria were purified by a Ficoll gradient ensuring a high degree of purity (see Supplementary methods online). Tyrosine phosphorylation is generally switched off under normal conditions, ascribable to the high activity of tyrosine phosphatases and to the general inactivation state of the tyrosine kinases in the absence of an appropriate signal [13]. Therefore, we treated RBM for 20 min at 30 °C with 1 mM peroxovanadate, a potent and generic tyrosine phosphatase inhibitor [14], to enhance the presentation of tyrosine phosphorylated proteins. The integrity of mitochondrial membranes during this treatment is mandatory to reduce the risk of introducing artifacts because of the peroxovanadate treatment. As reported in Supplementary methods, mitochondrial membrane potential and the respiratory control ratio do not change after the incubation. Moreover, we detected no cytochrome *c* by Western blotting in the supernatant after the treatment confirming the integrity of mitochondrial membranes (see Supplementary methods). Mitochondria were pelleted, lysed and tryptically digested. The phosphopeptides were then affinity captured using a mixture of 4G10 (Upstate Biotechnology) and PY20 (ICN) anti-phosphotyrosine antibodies. The extent of phosphorylation for untreated and treated samples was detected by anti-phosphotyrosine Western blotting as shown in Fig. 1A. Phosphopeptides were then analyzed by mass spectrometry. Initially, an enhanced multiple charge scan was used scanning primarily for doubly and triply charged ions. Despite detection of several phosphotyrosine-containing peptides, the majority of detected peptides were attributed to high abundant, non-phosphorylated peptides of mitochondrial proteins such as ATP synthase alpha/beta chain or malate dehydrogenase. By this approach seven phosphorylation sites were identified. To further improve this number, we used a precursor ion scan technique for the phosphotyrosine-immonium ion at *m/z* 216. This scan type exhibits some cross-talk to dipeptide ions at *m/z* 216 derived, e.g. from Asn-Thr, Gln-Ser or Lys-Ser. Nevertheless, the number of fragmented peptide signals was mainly limited to peptides containing the respective marker ion while omitting non-phosphorylated peptides still present after the affinity purification as background (compare Fig. 1B). This markedly increased the number of identified phosphopeptides and phosphorylation sites form 7 to 15, while covering all but one (Q62950 dihydropyrimidinase-related protein 1) of the sites initially detected without precursor ion scanning. High confidence in the identifications is based on four facts: (1) using a Mascot score cutoff for database search results of >35 equals to >99.5% probability for a true positive identification. (2) Searches against a concatenated Swiss-Prot decoy

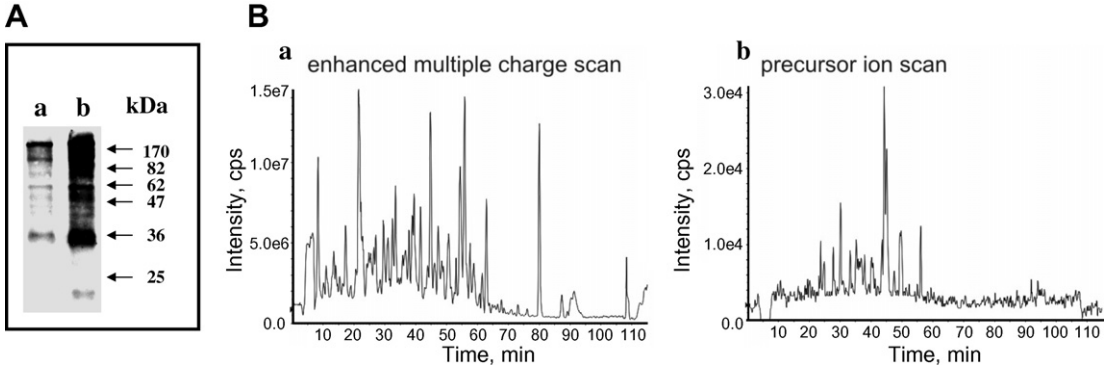

Fig. 1. (A) Anti-phosphotyrosine Western blotting of untreated control and peroxovanadate-treated RBM. Control (40 μg) (a) and treated RBM (b) were subjected to SDS–PAGE followed by Western Blotting on PVDF membrane and immunostaining with anti-phosphotyrosine antibody. (B) Base peak chromatograms of (a) enhanced multiple charge scans and (b) precursor ion scans for the analysis of affinity enriched phosphopeptides. The complexity of the sample mixture is clearly decreased by using a distinct selection criterion such as the phosphotyrosine-immonium ion at *m/z* 216. This enables the detection, fragmentation and identification even of low abundant phosphopeptides, whose signals are usually quenched in the presence of other peptide ion signals and thus not detected in common survey scans such as (a).