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The mitochondrial inner membrane protein Mitofilin exists as a complex with SAM50, metaxins 1 and 2, coiled-coil-helix coiled-coil-helix domain-containing protein 3 and 6 and DnaJC11

Jing Xie[a,*], Michael F. Marusich[b], Puneet Souda[c], Julian Whitelegge[c], Roderick A. Capaldi[a,b]

[a] *Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA*
[b] *MitoSciences, Inc., Eugene, OR, USA*
[c] *Department of Chemistry and Biochemistry, Molecular Biology Institute, UCLA, Los Angeles, CA, USA*

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Abstract A monoclonal antibody (mAb) has been produced which reacts with human mitofilin, a mitochondrial inner membrane protein. This mAb immunocaptures its target protein in association with six other proteins, metaxins 1 and 2, SAM50, CHCHD3, CHCHD6 and DnaJC11, respectively. The first three are outer membrane proteins, CHCHD3 has been assigned to the matrix space, and the other two proteins have not been described in mitochondria previously. The functional role of this new complex is uncertain. However, a role in protein import related to maintenance of mitochondrial structure is suggested as mitofilin helps regulate mitochondrial morphology and at least four of the associated proteins (metaxins 1 and 2, SAM50 and CHCHD3) have been implicated in protein import, while DnaJC11 is a chaperone-like protein that may have a similar role.

Keywords: Mitofilin; SAM50; Metaxin 1; Metaxin 2; CHCHD3; CHCHD6 DnaJC11; Monoclonal antibody; Mitochondria; Protein import; Sorting and assembly

1. Introduction

At one level, cellular processes are compartmentalized by their localization to specific organelles. In addition, it has become clear that these processes are further organized into multicomponent aggregates within which the product of one reaction is passed without release to become the substrate of the next enzyme/reaction. Examples include transcription, translation protein degradation, protein transport into organelles such as mitochondria and a number of metabolic pathways. Oxidative phosphorylation is one of the best characterized processes in which highly organized protein aggregates are involved. This important part of energy metabolism is catalyzed by electron transfer complexes I, II, III and IV, which are assemblies of 45, 4, 11 and 13 proteins, respectively [1]. These are further organized into super-complexes in the mitochondrial crystal membrane [2,3]. The end result is a highly organized multi-protein aggregate that optimally carries out a sequence of reactions with maximum conservancy of throughput by limiting competition for and/or protecting the structural integrity of intermediates generated during the overall process.

The individual electron transfer complexes represent tight associations of proteins that co-purify even under strong detergent or salt conditions [4]. This is not always the case with protein assemblies. A key to identifying any novel assembly is to be able to effectively sub-fractionate a cell or organellar extract under conditions mild enough to maintain relatively weak protein–protein interactions. Monoclonal antibody (mAb) immunoprecipitation is one way of accomplishing this. A mAb is made to a specific protein, the cell is disrupted, constituents solubilized, and the mAb used to immunocapture the antigen along with any associated proteins for identification by protein chemical methods.

Here we provide an example of using mAb immunocapture to identify novel protein associations in the cell. The work is a part of our ongoing efforts to generate mAbs against key proteins of mitochondria. We are using a shotgun method in which whole mitochondria are injected as an antigen mixture and mAbs capable of immunoprecipitating component proteins are sorted by rigorous screening that includes mass spectrometric identification of captured proteins after resolution in SDS–PAGE. Among the proteins immunoreactive in our study is mitofilin, a mitochondrial inner membrane protein thought to be involved in mitochondrial morphology maintenance [5]. Three independent mAbs reactive to mitofilin by Western blotting proved to immunocapture a complex of this protein along with a consistent set of six other mitochondrial proteins, including metaxins 1 and 2 [6], SAM50 [7] , coiled-coil-helix coiled-coil-helix domain 3 and 6 proteins [8,9] and the chaperonin protein DnaJC11[10]. The likely role of this complex in mitochondria is discussed.

2. Materials and methods

2.1. Human heart mitochondrial samples

Human heart mitochondria (HHM) purchased from Analytical Biological Systems, Inc. (Wilmington, DE) were prepared by tissue homogenization and differential centrifugation according to Pallotti and Lenaz [11] with minor modifications. The enriched mitochondria were used for immunization and following screen assays.

*Corresponding author. Fax: +1 541 284 1801.
E-mail address: cxjing@uoregon.edu (J. Xie).

Abbreviations: mAb, monoclonal antibody; OXPHOS, oxidative phosphorylation; HHM, muman heart mitochondria; LM, *n*-dodecyl-β-D-maltopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CM, conditioned media; MW, molecular weight

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2.2. Mitochondrial protein antibody production

HHM were used as antigen to immunize 3 CSJLF1/J strain mice. All of the procedures for hybridoma preparation were as described by Marusich [12]. Subsequent boosts were given at 3–4 week intervals and the final boosts were given at 4 and 3 days before the splenocytes were harvested. The immune lymphocytes from these mice spleens were then harvested and fused with myeloma cells to generate the hybridoma cell lines.

2.3. Solubulization of mitochondria proteins

Mitochondrial proteins were solubilized for immunocapture studies using 1% *n*-dodecyl-β-D-maltopyranoside (LM) as described previously [13].

2.4. Characterization of immunopurified proteins

Antibody bound beads were prepared as described in Ref. [14]. In a brief, 0.5 mg solubilized HHM was incubated overnight at 4 °C with individual conditioned media (CM) double coated protein G-agarose beads (#20398, Pierce). After the mixing step was complete, the CM specified antigens were eluted from the beads using 1% sodium dodecyl sulfate (SDS) and electrophoresed by 10–20% SDS–PAGE. All the bands of interest were excised manually for further mass spectrometry (MS) identification.

2.5. Sample preparation for mass spectrometry

Interested bands were excised and the in-gel protein samples were reduced, alkalized, and digested with 12.5 ng/μl trypsin over night at 37 °C. All procedures based on the standard protocol of UCLA Pasarow mass spectrometry laboratory. Digested peptides were collected and dried ready for MS analysis.

2.6. Analysis of peptides by nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS)

Trypsin digested peptides were analyzed by nLC-MS/MS with data-dependent acquisition (Q STAR XL; Applied Biosystems, Foster City, CA). Briefly, after dissolute in 10 μl of 0.1% formic acid, 5% acetonitrile (v/v) using either a split-flow HPLC (Ultimate, Dionex, CA) or a direct pumping HPLC (Eksigent, CA), samples were loaded to a trapping column (5 μL/min) previously equilibrated for 20 min with 100% A (A, 0.1% formic acid, 5% acetonitrile in water; B, 0.1% formic acid in acetonitrile) using an independent pump (Swithos on Ultimate or micropump on Eksigent). Ten minutes after sample loading the flow was switched (10-port valve, Switchos or Eksigent) such that the trap was back flushed by the nanoHPLC system. An analytical capillary reverse-phase column (75 μm × 10 cm; C18 5 μm, 300 Å; Microtech, CA) was equilibrated for 20 min at 300 nl/min with 100% A (A, 0.1% formic acid, 5% acetonitrile in water; B, 0.1% formic acid in acetonitrile) prior to the switching event and initiation of a compound linear gradient ramping to 80% A, 20% B at 8 min; 65% A, 35% B at 30 min; 25% A, 75% B at 40 min; and 100% A at 41 min. The analytical column flow was directed to a stainless steel nanoelectrospray emitter (ES301; Proxeon, Odense, Denmark) at 2.5 kV for ionization without nebulizer gas. The mass spectrometer was operated in information-dependent acquisition mode with a survey scan (*m*/*z* 400–1500), data-dependent MS/MS on the two most abundant ions with exclusion after two MS/MS experiments.

2.7. Data analysis

Individual sequencing experiments were matched to a global protein sequence database (MSDB) using Mascot software (Matrix Sciences, London, UK). The search was run under the "trypsin" mode to identify only tryptic peptides with fixed modification of Cys to its carboxyamidomethylated form (+57 Da). Variable modification of methionine to its sulfoxide (16 Da) was included. A significant probability score ($P < 0.05$ = MOWSE score > 63) was required for a secure identity assignment.

2.8. Sucrose gradient fractionation

To confirm the tight association of the newly identified mitofilin complex, and to determine whether or not the putative complex was an artifact due to mAb cross-reactivity to all proteins, sucrose gradient fractionation combined with Western blot and immune precipitation were performed. The distribution of the mitofilin complex in sucrose gradient fractions, and from this its approximate molecular weight, was determined by reference to that of the well known oxidative phosphorylation (OXPHOS) complexes [15]. In brief, solubilized HHM was loaded on the top of 4.5 ml of 15–35% sucrose gradient. After 16.5 h centrifugation at 128000 × *g* at 4 °C, the sucrose gradient was fractioned to 10 equal volume aliquots. The proteins in each fraction were then separated on 1D-SDS–PAGE gel. Mitofilin specific antibody (MM#7A-2E4AD5) was used as primary antibody to reveal location of the mitofilin complex. The OXPHOS complexes of mitochondria were blotted in parallel using the OXPHOS complex detection kit (#MS601, MitoSciences Inc. Eugene, OR). Western blotting was performed according to the manufacturer's recommended protocol. The mitofilin-enriched fractions were also further analyzed by immunocapture using the mitofilin antibody followed by SDS–PAGE.

3. Results

Human heart mitochondria were used as a mixture to generate mAbs to highly antigenic proteins of mitochondria. Among these were mAbs that immunocaptured each of the complexes of oxidative phosphorylation, proteins of apoptosis, Krebs cycle enzymes and enzymes of fatty acid oxidation. In addition to these previously known protein complexes, we identified mAbs that immunoprecipitate a novel multiprotein complex, which described as mitofilin complex here.

Three hybridomas generated mAbs that immunocaptured a protein complex showing the same set of component proteins of apparent molecular weight (MW) ranging from 83 kDa to 26 kDa. Gel bands were excised from SDS–PAGE gels and subjected to nLC-MS/MS. Fig. 1 shows the resolution of the several protein bands and lists the components in each band

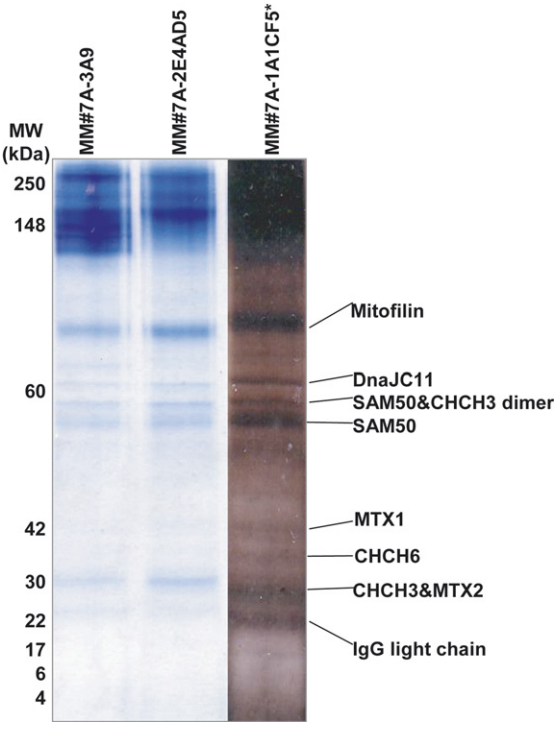

Fig. 1. Three newly generated mAbs specifically immunocaptured mitofilin along with six other proteins including SAM50 protein (sorting and assembly machinery component 50 homolog), MTX1&2 (metaxins 1 and 2), CHCHD3&6 (coiled-coil-helix coiled-coil-helix domain-containing protein 3 and 6), and DnaJC11 (DnaJ homolog subfamily C member 11). *Silver stain result.

Table 1
The protein identification result from MM#2E4AD5, 3A9 and 1A1CF5 using nLC-MS/MS

No.	Gene name	Protein name	MW	PI	AA	Mowse score	Sequence coverage (%)
M1	IMMT	mitofilin	82564	6.15	747	1596	44
M2	DNAJC11	DnaJ (Hsp40) homolog subfamily C member 11.	63278	8.54	559	839	30
M3	SAM50		51872	6.44	468	351	13
	CHCHD3	dimer	26136	8.48	227	302	18
M4	SAM50		51872	6.44	468	668	50
M5	MTX1	Metaxin-1	35754	5.9	317	316	22
M6	CHCHD6	Coiled-coil-helix coiled-coil-helix domain-containing protein 6	26441	9.01	235	255	30
M7	CHCHD3	Coiled-coil-helix coiled-coil-helix domain-containing protein 3	26136	8.48	227	583	26
M8	MTX2	Metaxin-2	29744	5.9	263	639	37
M9		IgG Lamda light chain	22547	5.79	213	222	17

from the mass spectrometry data (also see in Table 1). These components were consistently identified in the same bands in multiple immunoprecipitations and were the proteins mitofilin, DnaJC11, SAM50, metaxin 1, coiled-coil-helix coiled-coil-helix domain 6 protein (CHCHD6), coiled-coil-helix coiled-coil-helix domain 3 (CHCHD3) and metaxin 2 in order of molecular weight. To identify which protein in the complex was specifically recognized by each of the three mitofilin complex capture mAbs, Western blots were done using HHM as samples. In each case, the only band recognized was the 83 kDa mitofilin protein (Fig. 2a). To ensure the mitofilin monospecificity was not due to a lack of target material, the complex was first immunocaptured, and the immunoprecipitate subjected to Western blot analysis using the same capture mAb to probe the blot. As shown in Fig. 2b, the mAb again reacted monospecifically against mitofilin.

To confirm the tight association of the newly identified mitofilin complex in the absence of mAb binding, sucrose gradient fractionation was performed on solubilized HHM. This approach also provides a measure of the size of the mitofilin complex. Sucrose gradient fractionation separates protein complexes mainly by size but with a contribution from buoyant density related to lipid/protein/detergent ratios. Fig. 3 shows a typical result, with the five complexes of OXPHOS resolved across the gradient (identified by Western blotting). The mitofilin reactive mAb (applied during the Western blotting, not during running of the sucrose gradient) showed that the mitofilin-containing complex was eluted mainly in fractions 5, 6 and 7, thereby eluting as larger than complex II (MW 100 kDa) but smaller than F1ATPase (350 kDa) or the cytochrome *c* oxidase (Complex IV) dimer (400 kDa). This is consistent with a complex containing all six proteins with CHCHD3 as a dimer (aggregate MW 340 kDa). Finally, the anti-mitofilin mAb was used to immunocapture the complex from the three mitofilin-enriched sucrose gradient fractions i.e. from fractions 5, 6

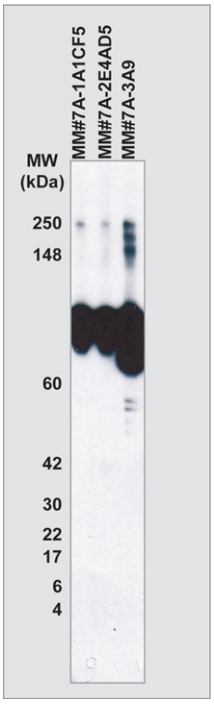

Fig. 2a. Using whole human heart mitochondria as sample, three newly generated mAbs recognized a single 83 kDa protein on Western blot identified by mass spectrometry as mitofilin.

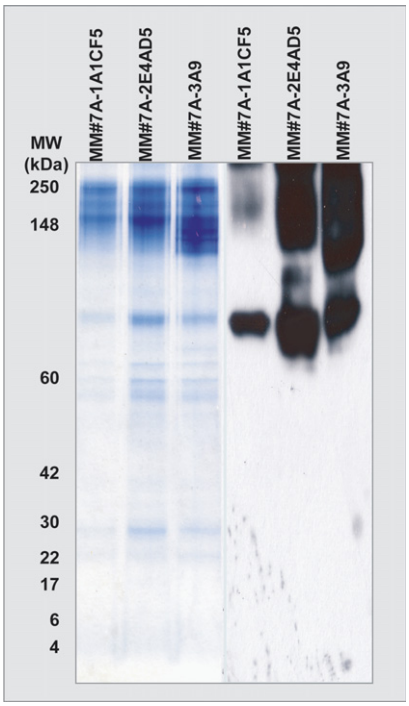

Fig. 2b. The left three lanes present the Coomassie stained immunoprecipitation result using the three new generated mitofilin mAbs. The right three lanes are the Western blot result using mitofilin mAb (2E4AD5) to blot the immunocaptured mitofilin complex, which confirms that mitofilin is the target antigen.

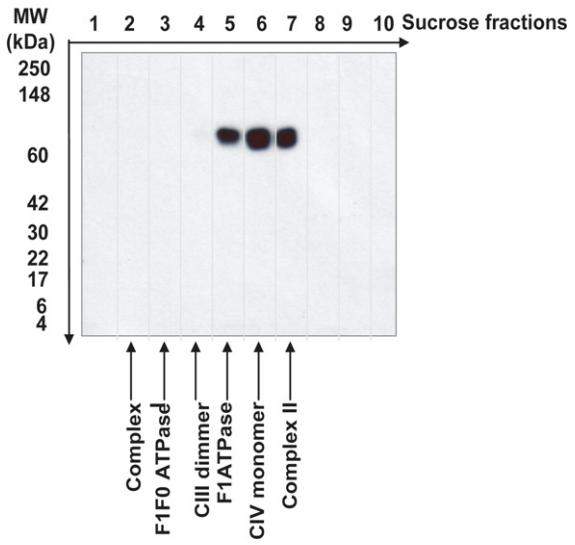

Fig. 3. The complex-relationship of mitofilin with the other five proteins was confirmed by sucrose gradient fractionation. The distribution of mitofilin was detected with newly generated antibody (2E4AD5) on PVDF membrane. The comparable distribution of OXPHOS complexes were blotted with total OXPHOS complex detection kit from MitoSciences Inc. The distribution of OXPHOS complex was labeled as reference. The mitofilin was further immune captured from its enriched sucrose gradient fractions (fraction 5–7). The immune purified proteins from each fraction repeated the identical gel pattern on 1D-SDS–PAGE as the one immunocaptured from the whole human heart mitochondria (see Fig. 1).

and 7. The result of this experiment (not shown) confirmed that anti-mitofilin mAb immunocaptured the same mitofilin complex containing all six proteins with the identical gel pattern to that when the complex was immunocaptured directly from the total mitochondrial protein sample.

4. Discussion

Mitofilin has been studied in several laboratories and characterized as an inner membrane protein with both coiled coil and inter-membrane space-targeting domains [16]. It is thought to function in mitochondrial cristae morphology [5]. This protein is reduced significantly in fetal Down's syndrome brain [17]. Present in association with mitofilin as immunocaptured here are so-called coiled-coil-helix coiled-coil-helix domain 3 and 6 proteins (CHCHD3 and CHCHD6) whose functions are poorly understood [8,9]. CHCHD3 has been identified before in mitochondria and tentatively assigned to the matrix space.

Three other proteins in this complex are metaxins 1 and 2 along with SAM 50 protein all of which have been characterized as outer membrane proteins. Metaxins 1 and 2 locate on the cytosol side of the outer membrane and serve in protein precursor import into mitochondria. Metaxin 2 is peripherally associates with mitochondrial outer membrane by means of its interaction with membrane-bound metaxin 1 protein. Neither of them associates directly with the TOM complex, but shares a limited homology with SAM37 protein, a subunit of the complex in the outer membrane responsible for beta-barrel protein import and assembly into the organelle [6,18,19]. Metaxin has been identified as a key factor in the tumor necrosis factor (TNF)-induced cell death [20]. SAM50 protein (sorting and assembly machinery component 50 homolog), the third component of outer membrane proteins in the complex, is also a beta-barrel protein and was shown to be required for incorporation of the TOM40 precursor into the mature TOM complex [21], in yeast, Sam50 is a core component of the SAM complex, which also includes SAM35 and SAM37 as subunits [7]. However, so far no homologue of Sam37 or Sam35 was identified in human samples. The most recent work of Kozjak-Pavlovic et al. observed that both Sam50 and metaxins 1 and 2 function in TOM40 and VDAC biogenesis [22]. The final protein identified as associated with mitofilin is DnaJC11 a protein identified only based on its gene sequence and a novel protein of mitochondria. DnaJC11 is one of the member proteins in 40 kDa heat shock protein subfamily C [23], and so may be a molecular chaperone during mitochondrial protein import and folding.

The immunocapture of the above proteins together by three independent anti-mitofilin mAbs and the co-elution of these proteins in sucrose gradient centrifugation provide strong but not definitive evidence of complex formation and it would be useful to have mAbs to components other than mitofilin for confirmatory immunocapture experiments but these reagents are not yet available. However, consistent with our results, John et al. reported that mitofilin is part of a supramolecular complex [5]. However, no proteomic analysis was done in that work to identify any non-mitofilin members of the complex.

In summary, our results emphasize the utility of the 'divide and conquer' approach we call focused proteomics for identifying mitochondrial proteins and protein–protein interactions. The approach has previously been used successfully to analyze the composition and post-translational modifications of known mitochondrial enzyme complexes (reviewed in [14,24]), and now has been used to identify the novel mitochondrial protein complex. In addition, two of the proteins described here as members of the putative mitofilin complex, DnaJC11 and CHCHD6, have not been identified previously with the organelle.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.06.052.

References

[1] Janssen, R.J., Nijtmans, L.G., van den Heuvel, L.P. and Smeitink, J.A. (2006) Mitochondrial complex I: structure, function and pathology. J. Inherit. Metab. Dis. 29, 499–515, Epub 2006 July 11.
[2] Boekema, E.J. and Braun, H.P. (2007) Supramolecular structure of the mitochondrial oxidative phosphorylation system. J. Biol. Chem. 282, 1–4, Epub 2006 Nov. 13.
[3] Gilkerson, R.W., Selker, J.M. and Capaldi, R.A. (2003) The crystal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Lett. 546, 355–358.
[4] Myasoedova, K.N., Arutyunyan, A.M. and Magretova, N.N. (2006) Phenomenon of activation of cytochrome P450 by nonionic detergents. Biosci. Rep. 26, 69–78.
[5] John, G.B., Shang, Y., et al. (2005) The mitochondrial inner membrane protein mitofilin controls cristae morphology. Mol. Biol. Cell 16, 1543–1554, Epub 2005 Jan. 12.

[6] Abdul, K.M., Terada, K., Yano, M., Ryan, M.T., Streimann, I., Hoogenraad, N.J. and Mori, M. (2000) Functional analysis of human metaxin in mitochondrial protein import in cultured cells and its relationship with the Tom complex. Biochem. Biophys. Res. Commun. 276, 1028–1034.
[7] Kozjak, V., Wiedemann, N., Milenkovic, D., Lohaus, C., Meyer, H.E., Guiard, B., Meisinger, C. and Pfanner, N. (2000) An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. J. Biol. Chem. 278, 48520–48523, Epub 2003 Oct. 21.
[8] Dreger, M., Mika, J., Bieller, A., Jahnel, R., Gillen, C., Schaefer, M.K., Weihe, E. and Hucho, F. (2005) Analysis of the dorsal spinal cord synaptic architecture by combined proteome analysis and in situ hybridization. J. Proteome Res. 4, 238–249.
[9] Gerhard, D.S., Wagner, L., et al. (2004) The status, quality, and expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC). Genome Res. 14, 2121–2127.
[10] Gregory, S.G., Barlow, K.F., et al. (2006) The DNA sequence and biological annotation of human chromosome 1. Nature 441, 315–321.
[11] Pallotti, F. and Lenaz, G. (2001) Isolation and subfractionation of mitochondria from animal cells and tissue culture lines. Methods Cell Biol. 65, 1–35.
[12] Marusich, M.F. (1998) Efficient hybridoma production using previously frozen splenocytes. J. Immunol. Methods 114, 155–159.
[13] Aggeler, R., Coons, J., Taylor, S.W., Ghosh, S.S., Garcia, J.J., Capaldi, R.A. and Marusich, M.F. (2002) A functionally active human F1F0 ATPase can be purified by immunocapture from heart tissue and fibroblast cell lines. Subunit structure and activity studies. J. Biol. Chem. 277, 33906–33912, Epub 2002 July 10.
[14] Murray, J., Yonally, S., Aggeler, R., Marusich, M.F. and Capaldi, R.A. (2004) Focused proteomics: towards a high throughput monoclonal antibody-based resolution of proteins for diagnosis of mitochondrial diseases. Biochim. Biophys. Acta 1659, 206–211.
[15] Taylor, S.W. et al. (2002) An alternative strategy to determine the mitochondrial proteome using sucrose gradient fractionation and 1D PAGE on highly purified human heart mitochondria. J. Proteome Res. 1, 451–458.
[16] Odgren, P.R., Toukatly, G., Bangs, P.L., Gilmore, R. and Fey, E.G. (1996) Molecular characterization of mitofilin (HMP), a mitochondria-associated protein with predicted coiled coil and intermembrane space targeting domains. J. Cell Sci. 109, 2253–2264.
[17] Myung, J.K., Gulesserian, T., Fountoulakis, M. and Lubec, G. (2003) Deranged hypothetical proteins Rik protein, Nit protein 2 and mitochondrial inner membrane protein, Mitofilin, in fetal Down syndrome brain. Cell. Mol. Biol. (Noisy-le-grand) 49, 739–746.
[18] Armstrong, L.C., Komiya, T., Bergman, B.E., Mihara, K. and Bornstein, P. (1997) Metaxin is a component of a preprotein import complex in the outer membrane of the mammalian mitochondrion. J. Biol. Chem. 272, 6510–6518.
[19] Armstrong, L.C., Saenz, A.J. and Bornstein, P. (1999) Metaxin 1 interacts with metaxin 2, a novel related protein associated with the mammalian mitochondrial outer membrane. J. Cell. Biochem. 74, 11–22.
[20] Wang, X., Ono, K., Kim, S.O., Kravchenko, V., Lin, S.C. and Han, J. (2001) Metaxin is required for tumor necrosis factor-induced cell death. EMBO Rep. 2, 628–633, Epub 2001 July 3.
[21] Humphries, A.D., Streimann, I.C., Stojanovski, D., Johnston, A.J., Yano, M., Hoogenraad, N.J. and Ryan, M.T. (2005) Dissection of the mitochondrial import and assembly pathway for human Tom40. J. Biol. Chem. 280, 11535–11543, Epub 2005 Jan. 10.
[22] Kozjak-Pavlovic, V., Ross, K., Benlasfer, N., Kimmig, S., Karlas, A. and Rudel, T. (2007) Conserved roles of Sam50 and metaxins in VDAC biogenesis. EMBO Rep. 18, 18.
[23] See http://www.expasy.org/cgi-bin/get-sprot-entry?Q9NVH1.
[24] Murray, J., Marusich, M.F., Capaldi, R.A. and Aggeler, R. (2004) Focused proteomics: monoclonal antibody-based isolation of the oxidative phosphorylation machinery and detection of phosphoproteins using a fluorescent phosphoprotein gel stain. Electrophoresis 25, 2520–2525.