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Topological analysis of ATAD3A insertion in purified human mitochondria

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Abstract ATAD3 is a mitochondrial inner membrane-associated protein that has been predicted to be an ATPase but from which no associated function is known. The topology of ATAD3 in mitochondrial membranes is not clear and subject to controversy. A direct interaction of the N-terminal domain (amino-acids 44–247) with the mtDNA has been described, but the same domain has been reported to be sensitive to limited proteolysis in purified mitochondria. Furthermore, ATAD3 has been found in a large purified nucleoid complex but could not be cross-linked to the nucleoid. To resolve these discrepancies we used two immunological approaches to test whether the N-terminal (amino-acids 40–53) and the C-terminal (amino-acids 572–586) regions of ATAD3 are accessible from the cytosol. Using N-terminal and C-terminal specific anti-peptide antibodies, we carried out back-titration ELISA measurements and immunofluorescence analysis on freshly purified human mitochondria. Both approaches showed that the N-terminal region of ATAD3A is accessible to antibodies in purified mitochondria. The N-terminal region of ATAD3A is thus probably in the cytoplasm or in an accessible intermembrane space. On the contrary, the C-terminal region is not accessible to the antibody and is probably located within the matrix. These results demonstrate both that the N-terminal part of ATAD3A is outside the inner membrane and that the C-terminal part is inside the matrix.

Keywords ATAD3 · tob3 · Mitochondrial AAA-ATPase

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Introduction

ATAD3 (ATPase family AAA domain-containing protein 3) is a 68 kDa (586 amino-acids) mitochondrial protein that has been predicted to be an ATPase but whose function is still unknown. The atad3 gene, not present in yeast, appears in *Caenorhabditis elegans*, *Arabidopsis thaliana* and *Drosophila melanogaster* (called belphegor) as a single gene. The gene has remained unique in vertebrate and monkeys and a second gene, contiguous on the distal 1p chromosome arm, has appeared in human (this gene is called atad3B, versus atad3A the ancestral form). Atad3 gene was first described as a target of c-Myc via a large screening for c-Myc transactivated genes (Zeller et al. 2003). ATAD3 protein was later identified in patients with carcinoma as an antigen arising from the auto-immune response (Schaffrik et al. 2006; Gires et al. 2004). ATAD3 was also found overexpressed in these carcinomas and its localization was assessed to be mitochondrial (Schaffrik et al. 2006). Proteomic approaches from murine tissues identified ATAD3 as a protein associated with the inner membrane (Da Cruz and Martinou 2008; Da Cruz et al. 2003). It has been also shown to be essential for early embryogenesis in *Caenorhabditis elegans* (Piano et al. 2002; Kamath and Ahringer 2003; Kamath et al. 2003; Simmer et

al. 2003; Sonnichsen et al. 2005; Hoffmann et al. 2009). The protein may therefore be involved in an essential mitochondrial function existing since pluricellular organization and developed in humans.

An improved understanding of ATAD3 localization and topology would contribute to discovering its cellular function and partners. The analysis of the primary sequence of ATAD3 reveals two major domains. The C-terminal region (337–586) constitutes the ATPase core with the well-known Walker's sequences. The specific N-terminal region (1–336) contains few putative transmembrane domains, a flexible proline-rich region and a coiled-coil region. Among these putative transmembrane domains, one, predicted upon several different approaches, may be relevant. However, and because of these others putative transmembrane domains, the topology of ATAD3 in mitochondrial membranes has not been determined and requires clarification (N.B. ATAD3 does not possess a canonical mitochondrial targeting sequence). Previous experiments have shown that ATAD3 is an integral protein due to its resistance to detergent or salt extractions (He et al. 2007). In the same article, He and collaborators propose the possible existence of a N-terminal interaction of ATAD3 with mitochondrial DNA. However, nucleoid proteomic approaches did not confirm such a direct interaction (Bogenhagen et al. 2008). Furthermore, proteolysis experiments on fresh mitochondria have shown that the N-terminal domain is rapidly and totally digested and thus probably exposed to the cytosol or to an accessible intermembrane space. On the contrary, the C-terminal domain is resistant to proteolysis and thus probably in the matrix (Bogenhagen et al. 2008).

To address the question of the topology of ATAD3 in the mitochondrial membranes, we undertook two immuno-topological approaches on purified human mitochondria from the HS683 cell line, previously shown to express only ATAD3A (Hubstenberger et al. 2008). We performed back-titration ELISA and immuno-fluorescence analysis on purified human mitochondria using two anti-peptide antibodies directed against the N-terminal (amino-acids 40–53) and the C-terminal (amino-acids 572–586) regions of ATAD3A.

Both approaches sustain the hypothesis that the N-terminal part of ATAD3A is exposed to the outside of the inner membrane whereas the C-terminal part is in the matrix.

Materials and methods

Cell culture

HS683 cells (derived from a 76 year old male's oligodendroglioma, HTB-138, ATCC) were cultured as monolayers in Dulbecco's Minimal Essential Medium (Gibco BRL, DMEM-Glutamax) supplemented with 10% fetal calf serum. The cells were incubated in a 37 °C incubator in an atmosphere of 5% CO_2.

Antibodies

Anti N- and C-terminal antibodies against ATAD3 were obtained from rabbit immunization with the following peptides: N-terminal (R40PAPKDKWSNFDPTG53) and C-terminal (L572KAEGPGRGDEPSPS586). Anti-peptide antibodies were purified against the peptide used for immunization and used at a 1/1,000 dilution (Eurogenetec). Anti-ANT1, Lamin A&C, Tubulin, cytochrome C and Tom20 antibodies are polyclonal antibodies raised in rabbits with the whole human protein form.

Mitochondria preparation

From subconfluent cultures, cells were rinsed in PBS, recovered in PBS-PMSF 1 mM using a Rubber Policeman, pelleted (1,000 g/ 10 min) and lysed with a syringe (22 g needle) in lysis buffer (Sucrose 10% (m/v), Tris 10 mM pH 7,4, EDTA 1 mM, PMSF 1 mM). This step was controlled by microscopy to reach 99% of cell lysis. The lysate was centrifuged to pellet nuclei and unlysed cells and mitochondria from the supernatant were recovered by centrifugation (10,000 g, 20 min). Sonication was done at 10 W for 10 min at 4 °C (Bioblock Scientific VibraCell). Protein concentration was determined by micro-BCA™ (ECL Pierce). Mitochondrial membrane's integrity was checked by pre-incubating separated fractions with Mitotracker Red CM-H2XRos (579 nm, Molecular Probes), a membrane potential-sensitive dye, before mounting and visualizing them by confocal microscopy. For trypsin digestion, mitochondria (500 μg) were incubated with trypsin (5 μg) for various times at 37 °C in 10 mM Tris pH8, 0.12 M KCl. Reaction was stopped with SDS loading buffer and protein extracts were analyzed by Western-blot.

Western-blot

Human HS683 cells or cellular sub-fractions were lysed in SDS-sample buffer. Proteins were run on 9% acrylamide SDS-PAGE gel, and transferred onto nitrocellulose membrane. Immunoblotting was performed in PBS-0.2% Tween 20 and then incubated with the different antibodies revealed by chemoluminescence detection of peroxydase-coupled secondary antibodies (Pierce).

ELISA

Anti-peptide antibodies specificity was assayed by pre-incubating them overnight with various peptides. Anti-

bodies were then incubated with mitochondria coated on ELISA plates (polysorp, NUNC), 2 h at 4 °C. After rinsing with PBS (3 times), plated mitochondria were incubated at room temperature for 2 h with the secondary antibodies (anti-Goat IgG-peroxydase conjugate), rinsed and incubated with ELISA staining buffer (Sodium Acetate 100 mM, pH 6 tetramethylbenzidine 0.1 g/l, H_20_2 0,005% (v/v)). The colorimetric reaction was stopped using sulphuric acid (2 M) and the absorbance was read at 450 nm (Thermo Multiskan). For the other back-titration ELISAs, antibodies were pre-incubated overnight at 4 °C with either sonicated or intact mitochondria (Brandolin et al. 1989).

Immunofluorescence

Purified mitochondria were incubated overnight with primary antibodies at 4 °C in PBS. After rinsing with PBS (3 times), the mitochondria were incubated with secondary antibodies coupled to flurochromes (642 nm, Fluoprobe) and Mitotracker Green FM (488 nm, Molecular Probes) for 2 h at 4 °C. Mitochondria were then washed with PBS (3 times) and mounted on slides with anti-bleaching medium (DEBCO, glycerol 20% (m/v)). Preparations were imaged using confocal microscope (LEICA). For mitochondria permeabilization, mitochondria were treated with 70% cold ethanol for 15 min at 4 °C, rinsed 15 min with PBS-Triton X100 0,1% prior to immuno-detection.

Results

Putative topology of ATAD3A

Biostatistic analysis of the primary sequence of ATAD3A (Q9NVI7-2) showed two major domains (cf Fig. 1). The C-terminal part (aa 337–586) makes up the ATPase core domain with the well-known Walker's sequence. The N-terminal part (aa 1–336) contains a flexible proline-rich region (aa 18–27, http://smart.embl.de/smart/show_motif.pl), a coiled-coil region (aa 80–220, http://www.ch.embnet.org/cgi-bin/COILS_form.html) and a few putative transmembrane domains. Searches for transmembrane domains using Smart, SOSUI and TMHMM softwares were inconclusive. The DAS and TOPpred programs detected one transmembrane domain (aa 249–264 or 242–262 respectively), TMpred program identified two transmembrane domains (aa 243–260 and 346–366), whereas HMMTOP program identified three transmembrane domains (aa 220–248, 242–250 and 343–363) (cf Fig. 1). Among these putative transmembrane domains, the central one was always predicted (aa 249–264). In view of these predictions, one can hypothesize

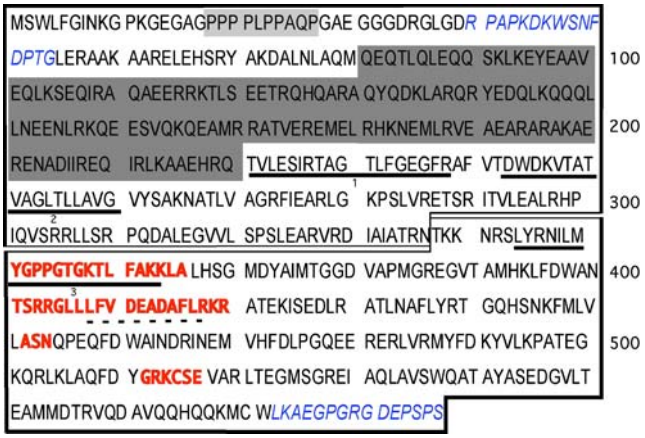

Fig. 1 Primary sequence of human ATAD3A. ATAD3A is composed of a N-terminal specific domain (a.a.1–336) and a C-terminal ATPase core domain (a.a.337–586,). The proline-rich stretch is *boxed* (a.a. 18–27, *light grey*), the Walker's sites are in *bold* (by order, Walker A, (YGPPGTGKTLFAKKLA) or AA 351-366, Walker B (TSRRGLLLFVDEADAFLRKR) or AA 401-420, sensor I and sensor II) and the three putative transmembrane domains are *underlined* and *numbered*. The coiled-coil structure is *boxed* (a.a. 80–220, *dark grey*) and the DEAD box is *dotted underlined*. N-terminal (R40PAPKDKWSNFDPTG53) and C-terminal (L572KAEGPGRGDEPSPS586) peptides used to raise anti-peptide antibodies are in *italics*

that the ATAD3 molecule spans the outer and/or the inner mitochondrial membrane at least once.

Control of mitochondrial fractionation quality

Our goal being the exploration of the ATAD3A topology in human purified mitochondria, we carried out conventional mitochondria purification from HS683 human glioma cells, previously shown to express only ATAD3A (Hubstenberger et al. 2008). The quality of the fractionation was controlled by the analysis of sub-cellular markers by Western-blot (Fig. 2a). As it can be seen, the mitochondrial sub-fraction was free of nuclear and cytoplasmic contaminants (lamin and tubulin respectively). No traces of mitochondrial membranes were detected in the cytoplasm (ANT1, Adenine Nucleotide Transporter 1). ATAD3A was detected as a single band, with both antibodies, only in the mitochondrial fraction. No major unspecific signal was seen.

To evaluate the integrity of purified mitochondria, we used the Red CM-H2XRos Mitotracker to stain polarized mitochondria and observed them by confocal microscopy (Fig. 2b). As shown, the mitochondrial subfraction was composed of 1 μM mean size circular and tubular particles. Under our conditions of purification, about 99% of the mitochondria were highly stained and consequently polarized and not leaky. We also performed a trypsin proteolysis of these mitochondria and found, as previously described under these conditions (Bogenhagen et al. 2008), that the

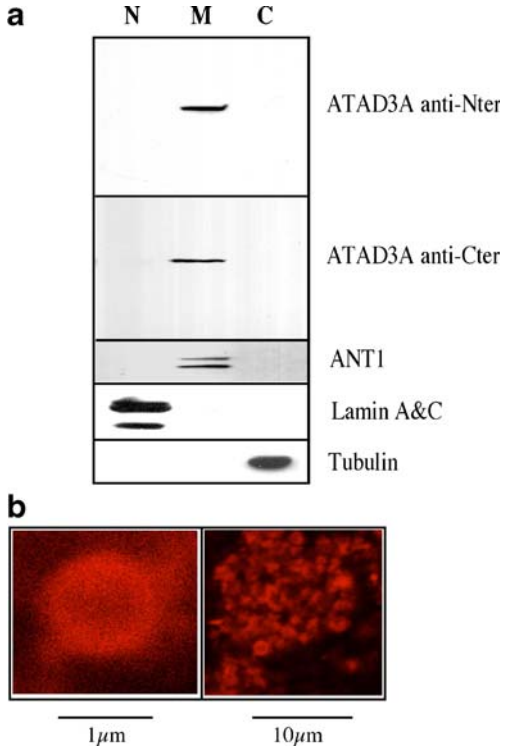

Fig. 2 Control of the mitochondrial fractionation quality. **a** Mitochondria from human HS683 cells were purified and nuclear (N), cytoplasmic (C) and mitochondrial (M) sub-fractions were analyzed by western-blot with anti-ATAD3A (anti-Nter and anti-Cter), anti-ANT1, anti-Lamin A&C and anti-Tubulin antibodies. **b** Purified mitochondria were stained with the Red CM-H2XRos Mitotracker and observed by confocal microscopy. Scales are indicated

N-terminal part of ATAD3A is rapidly digested, leaving a 40 kDa C-terminal domain resistant to proteolysis (Fig. 3). As controls, we used ANT1 (inner membrane), Cytochrome C (inter membrane space) and Tom 20 (outer membrane).

Specificity of ATAD3A detection by ELISA on purified mitochondria

The rationale of our approach was to use two anti-peptide antibodies to analyze the topology of ATAD3A in freshly purified human mitochondria. These antibodies were obtained from rabbit immunization with the peptides, RPAPKDKWSNFDPTG corresponding to the N-terminal part of ATAD3A (a.a. 40–53), and LKAEGPGRDEPSPS corresponding to the C-terminal part (a.a. 572–586) (Fig. 1).

The specificity of ATAD3A immuno-detection in purified mitochondria was tested by direct ELISA with mitochondria (intact or sonicated) coated on plates and with antibodies pre-incubated with increasing amounts of the peptide used for immunization.

Since it gave a higher signal than sonicated mitochondria (data not shown), the response of the anti-N-terminal antibody was measured by ELISA using intact mitochondria. As shown in Fig. 4a, increasing amounts of competing N-terminal peptide inhibited the immuno-detection in a dose-dependent manner. As expected, when the C-terminal peptide was used at a similar concentration it had no effect.

Similarly, the specific immuno-reactivity of the anti-C-terminal antibody was measured by ELISA with coated sonicated mitochondria since intact mitochondria did not yield significant signal (data not shown). As shown in Fig. 4b, increasing amounts of competing C-terminal peptide resulted in a decrease in the signal in a dose-dependent manner. As expected at a similar concentration, the N-terminal peptide produced no detectable effect. Taken together, these results demonstrate the specificity of ATAD3A ELISA-detection on purified mitochondria with either N-terminal or C-terminal antibodies.

ATAD3A accessibility measured by back-titration ELISA

In order to assess the localization of the N- and C-termini of ATAD3A on either the internal or external faces of mitochondrial membranes, we compared the accessibility of the N- and C-terminal regions of ATAD3A in both intact mitochondria and sonicated mitochondria (which induces membranes inversion, randomly 50% inside-out). Direct ELISA measurements of ATAD3A in intact and sonicated mitochondria are not directly comparable because the coating efficiency is different (usually two times greater with intact mitochondria). Therefore, we carried out back-titration ELISA in which intact or sonicated mitochondria are first incubated in suspension with antibodies (Brandolin

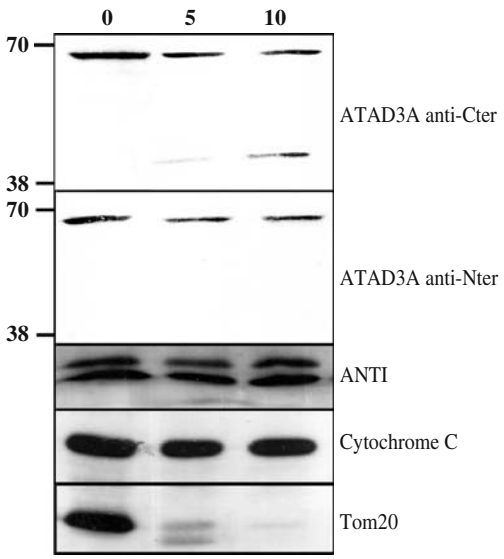

Fig. 3 Proteolytic digestion of purified mitochondria. Purified mitochondria were incubated with trypsin at 37 °C for various time from 0 to 10 min and analyzed by Western-blot with anti-ATAD3 anti-peptide antibodies (anti-Nter and anti-Cter), anti-Tom20, anti-ANT1 and anti-Cytochrome C polyclonal antibodies

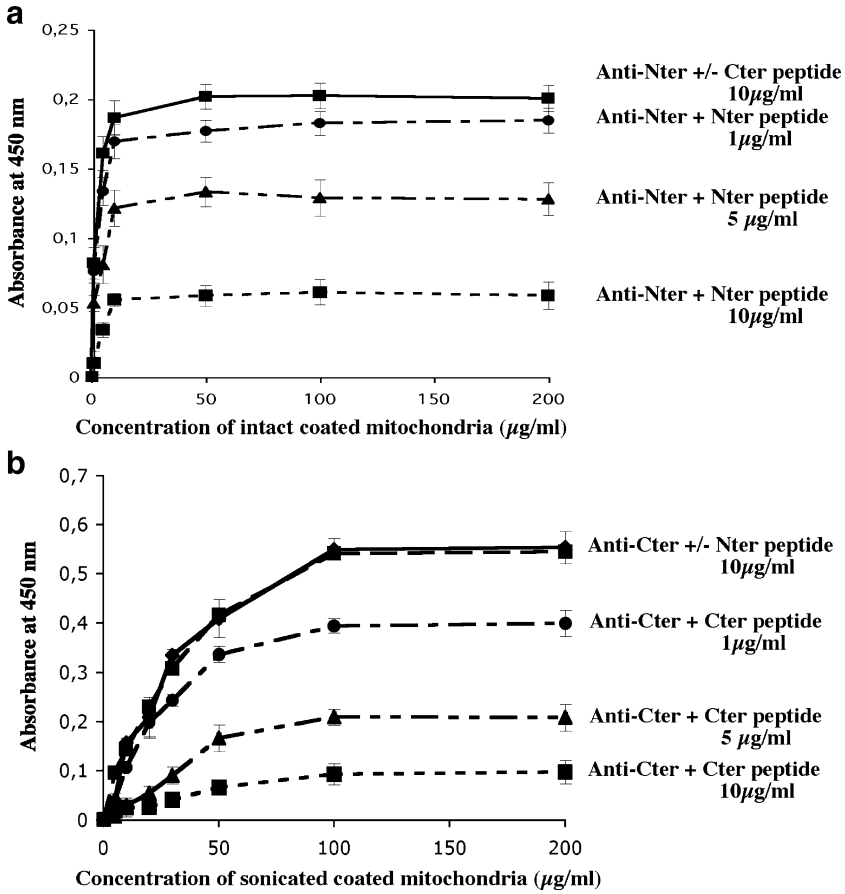

Fig. 4 Specificity of ATAD3A-ELISA detection on purified mitochondria. **a** Purified mitochondria from HS683 cells were coated on 96-well ELISA plates and the anti-Nter antibody was pre-incubated without (−) or with (+) increasing amounts of the N-terminus peptide (0, 1, 5 and 10 μg/ml) and C-terminus peptide (10 μg/ml) prior to incubation on the wells. Immuno-complex formation was detected by peroxidase activity measured by the absorbance at 450 nm. **b** Purified sonicated mitochondria from HS683 cells were coated on 96-well ELISA plates and the anti-Cter antibody was pre-incubated with increasing amounts of C-terminus peptide (0, 1, 5 and 10 μg/ml) and N-terminus peptide (10 μg/ml). Immuno-complex formation was detected by peroxidase activity measured by the absorbance at 450 nm

et al. 1989). Following this the unreacted anti-Nter or anti-Cter antibodies were titrated in an ELISA test with increasing amounts of mitochondria coated on plates.

The N-terminus accessibility was tested after pre-incubation of the anti-N-terminal antibody with increasing amounts of intact or sonicated mitochondria. The percentages of inhibition obtained are shown in Fig. 5a. We observed that intact mitochondria are more reactive to the antibody than inverted material. Titration by sonicated mitochondria reached 50%, corresponding to the theorical rate of membrane inversion. These results are consistent with exposure of the N-terminus epitope outside of the matrix (accessible to the antibody), to the external face of the mitochondria. Hence, membrane inversion induced by sonication results in the hiding of this region.

Similar experiments were performed to determine the C-terminus epitope accessibility. In this case, as intact mitochondria gave no observable signal in direct ELISA (data not shown), we coated sonicated mitochondria on the wells and pre-incubated the anti-C-terminal antibody with increasing amounts of intact or sonicated mitochondria. The percentages of inhibition are shown in Fig. 5b. We observed that sonicated mitochondria reacted with the antibody better than with intact mitochondria, meaning that the C-terminus epitope of ATAD3A had become unmasked upon sonication. However, the titration did not attain 100% probably because the plateau is not reached. Also, intact mitochondria reacted with the C-ter antibody (20% inhibition). Hence, and because direct ELISA detection of ATAD3A with the C-ter antibody did not give any significant results, an unidentified background may exist in this back-titration ELISA assay.

ATAD3A accessibility revealed by immuno-fluorescence

To confirm our results, we performed immuno-fluorescence analysis on purified mitochondria. Freshly purified mitochondria were incubated, in the absence of both detergent

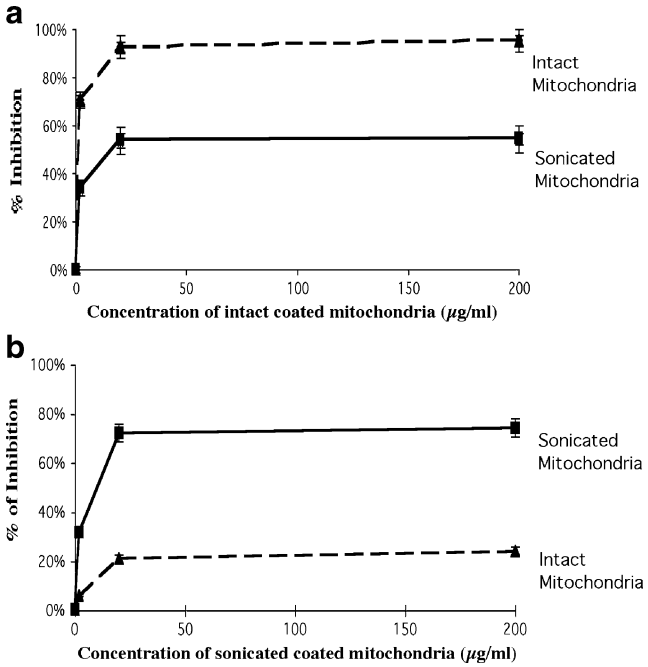

Fig. 5 Measurement of ATAD3A accessibility in purified mitochondria by back-titration ELISA. **a** Purified intact mitochondria from HS683 cells were coated on 96-well ELISA plates and the anti-Nter antibody pre-incubated with increasing amounts of intact or sonicated mitochondria prior to the incubation in the wells. Immuno-complex formation was detected by peroxidase activity measured by the absorbance at 450 nm. **b** Purified sonicated mitochondria from HS683 cells were coated on 96-well ELISA plates and the anti-Cter antibody pre-incubated with increasing amounts of intact or sonicated mitochondria prior to the incubation in the wells. Immuno-complex formation was detected by peroxidase activity measured by the absorbance at 450 nm

and fixer, with anti-Nter and anti-Cter antibodies and revealed by a fluorescent secondary antibody. The Green-Mitotracker was used to co-stain the mitochondria. Under native condition, the anti-Nter antibody strongly recognized purified mitochondria and co-localized with the mitochondrial-membranes Mitotracker staining (Fig. 6). On the contrary, the anti-Cter antibody did not yield any significant signal. To check that the anti-Cter antibody is capable of recognizing ATAD3A by immuno-fluorescence, we permeabilized mitochondria with 70% ethanol prior to immuno-detection. As shown in Fig. 6, this allowed detection of the ATAD3A C-terminus epitope (and of N-terminus epitope, data not shown). As a control, we used an anti-ANT1 polyclonal antibody that should not detect ANT1 in intact mitochondria. As can be seen in Fig. 6, ANT1 is not detected in purified mitochondria. As for ATAD3A C-terminus epitope, permeabilization of mitochondria with ethanol allowed ANT1 to be stained, demonstrating both that immuno-detection of ANT1 and ATAD3A C-terminal epitope requires permeabilization and that the purified mitochondria remain essentially intact.

Discussion

Knowledge of how the ATAD3A molecule is inserted in the mitochondrial membranes is basic to understanding its function and seeking cellular partners. Using two immunological approaches (ELISA and immuno-fluorescence microscopy) involving N-terminal (amino-acids 40–53) and C-terminal (amino-acids 572–586) anti-peptide antibodies, we show that the N-terminal epitope of ATAD3A is recognized by the anti-N-terminal antibody on freshly purified mitochondria. On the contrary, the C-terminal epitope is detected only after sonication or permeabilization of purified mitochondria while the N-terminal epitope remains accessible. This indicates both that the N-terminal region is located outside the inner mitochondrial membrane, facing the cytosol or the intermembrane space (see below), and that the C-terminal region is oriented toward the matrix. These deductions contradict the conclusions of He and colleagues who showed that the N-terminal part of ATAD3 (44–247) could interact with mitochondrial DNA (He et al. 2007). It should be noted that ATAD3 is a basic (Isoelectric point: 9) and abundant protein (around 5 times less than ANT1) that can non-specifically associate with cellular components thus leading to contaminated fractions.

The initial search for nucleoid-associated components raised the possibility that ATAD3 might be adjacent to the nucleoid (Wang and Bogenhagen 2006). However, a clearer nucleoid proteomic approach, with cross-linking experiments did not confirm this possibility (Bogenhagen et al. 2008). In addition, proteolysis experiments carried out on freshly isolated mitochondria show that the N-terminal domain is rapidly digested and then probably exposed to the cytosol or to accessible intermembrane spaces (Bogenhagen et al. 2008). This uncertainty is due to the fact that trypsin digestion of isolated mitochondria may induce outer membrane permeability to trypsin itself. It is therefore difficult to believe that the N-terminal part of the ATAD3A molecule interacts with the nucleoid. Following our observations, it seems clear that the N-terminal part of ATAD3A is, at least in part, located outside of the mitochondria. One explanation that could reconcile these contradictory observations is that ATAD3A exists in two conformations, in which the N-terminal part is exposed to either the inside or the outside of the mitochondria. However, such a hypothesis is not consistent with proteolysis experiments showing that the N-terminal part is totally digested, with no resistant form detected. This unambiguously shows that the N-terminal region of ATAD3A is uniformly located outside the mitochondrial matrix. Nevertheless, we cannot rule out the possibility that purified mitochondria are totally intact, especially regarding the leakiness of the external membrane. Therefore, we believe that the N-terminal part

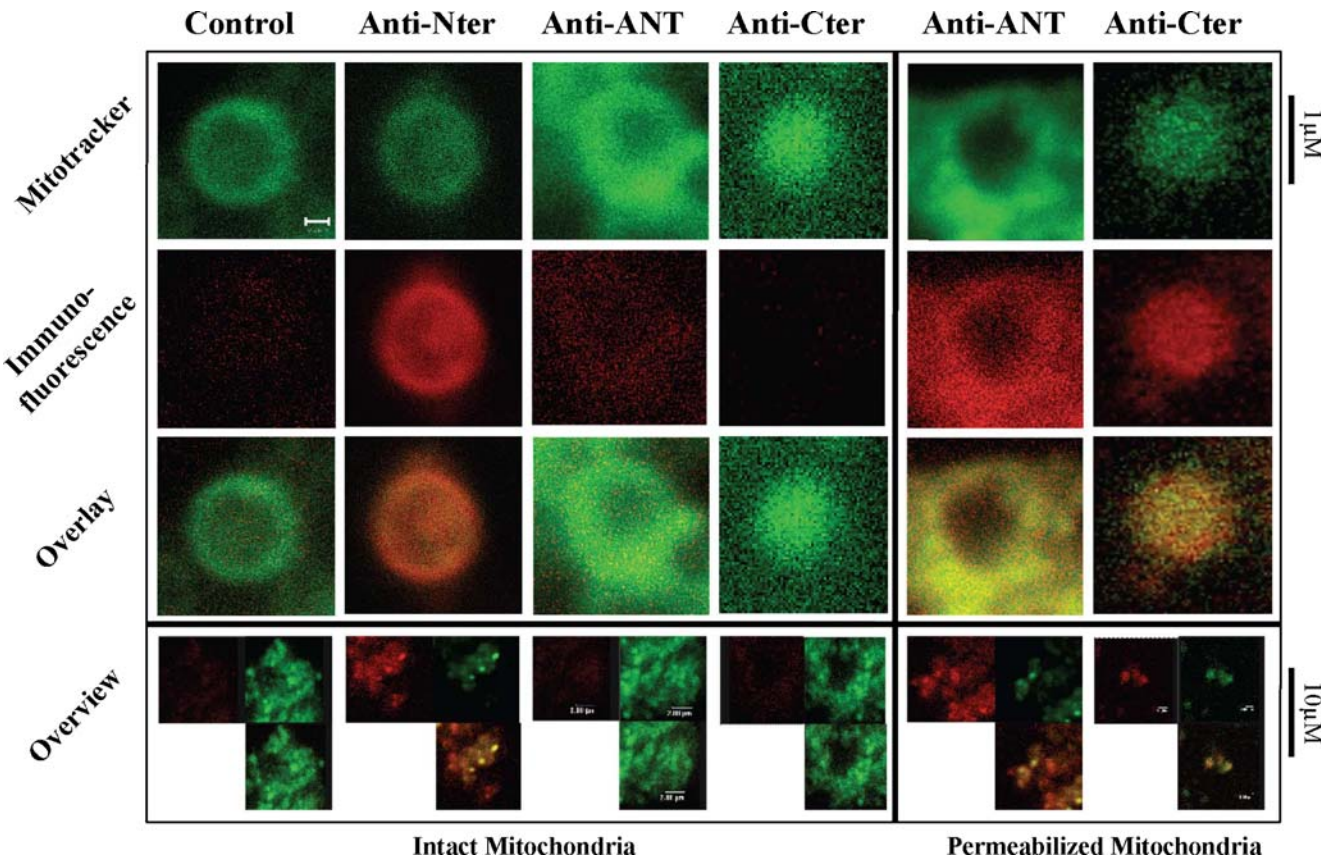

Fig. 6 ATAD3A detection by immuno-fluorescence on purified mitochondria. Purified mitochondria from HS683 cells were immuno-stained for ATAD3A, using both anti N-ter and anti-Cter antibodies and for ANT1. Results obtained with intact mitochondria are presented on the *left* and results obtained with permeabilized mitochondria are presented on the *right*. Immuno-staining is in *red* and Mitotracker in *green*. Scales are indicated

of ATAD3A is located in the intermembrane space, as suggested by Bogenhagen et al., or in the cytosol.

Concerning the C-terminal region of ATAD3A, we failed to detect any immuno-fluorescent or ELISA signal with purified mitochondria. This indicates that this region is not accessible from outside the mitochondria. However, back-titration ELISA approach did not completely confirm this result since mitochondria displayed some reactivity toward the anti C-ter antibodies. This discrepancy could be explained by the detection sensitivity of the method we used, and the existence of a non-specific background. However, sonication of mitochondria increased the immuno-detection of the C-terminal part showing that some ATAD3A C-terminal regions, if not all, are not accessible in purified mitochondria, and are probably facing the matrix.

In order to have the C-terminal part oriented toward the matrix and the N-terminal part facing the cytosol, the ATAD3A molecule should have both outer and inner transmembrane domains. Figure 1 and 7 illustrates that this is theoretically possible. However, no protein spanning both membranes has ever been described excepted for some steps of the import process of nuclear-encoded proteins in

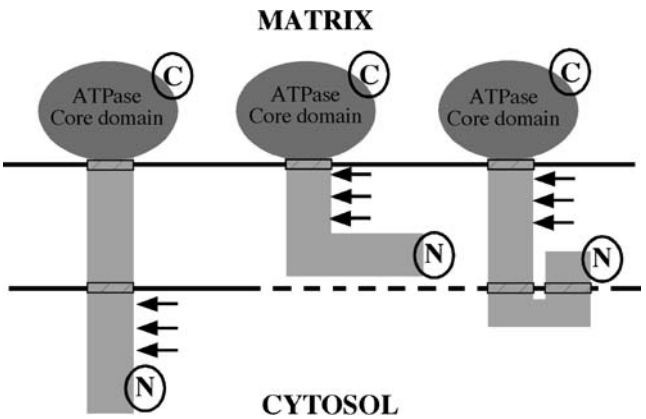

Fig. 7 Schematic overview of ATAD3A topology. Three different insertions are described, as discussed, all showing the C-terminal ATPase core domain inside the matrix. The N-terminal segment, accessible to both trypsin and antibodies, is either in the cytosol or in the inter-membrane space, considering that ATAD3A has one to three transmembrane domains (*lined rectangles*). The N-ter and C-ter epitope are evoked and the trypsin sensitivity is indicated by *arrows*

mitochondria. Therefore, it is more likely that the C-terminal part of ATAD3A is located in the matrix and the N-terminal domain in the intermembrane space (Fig. 7). This seems possible as ATAD3A is thought to be in tight association with the inner membrane as shown by its resistance to alkaline carbonate lysis (He et al. 2007 and Da Cruz et al. 2003). A more detailed study, for example by electron microscopy, is necessary to answer this question.

The topology of ATAD3B, the second human paralogue, is presently under progress, using specific antibodies. The ATAD3B molecule contains the same putative transmembrane domains as ATAD3A molecules, plus in addition another in the C-terminal domain (606–630) that may permit a partial localization either in the cytosol or the intermembrane space. The strong homology of the primary sequences (93% identity) of the two molecules suggests that they have the same location in the membrane. However, ATAD3B contains a C-terminal stretch rich in cysteines which may confer different biochemical properties.

Finally, it should be noted that although a splicing variant of ATAD3A has been observed (Q9NVI7-2, 72kD) but it is not present in our cells. However, this additional region of 48 amino acids (95–142) contains no potential transmembrane domains.

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References

Bogenhagen DF, Rousseau D, Burke S (2008) The layered structure of human mitochondrial DNA nucleoids. J Biol Chem 283(6):3665–3675

Brandolin G, Boulay F, Dalbon P, Vignais PV (1989) Orientation of the N-terminal region of ANT1. Biochemistry 28:1093–1100

Da Cruz S, Martinou JC (2008) Purification and proteomic analysis of the mouse liver mitochondrial inner membrane. Methods Mol 432:101–116

Da Cruz S, Xenarios I, Langridge J, Vilbois F, Parone PA, Martinou JC (2003) Proteomic analysis of the mouse liver mitochondrial inner membrane. J Biol Chem 278(42):41566–41571

Gires O, Münz M, Schaffrik M, Kieu C, Rauch J, Ahlemann M, Eberle D, Mack B, Wollenberg B, Lang S, Hofmann T, Hammerschmidt W, Zeidler R (2004) Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology. Cell Mol Life Sci 61(10):1198–1207

He J, Mao CC, Reyes A, Sembongi H, Di Re M, Granycome C, Clippingdale AB, Fearnley IM, Harbour M, Robinson AJ, Reichelt S, Spelbrink JN, Walker JE, Holt IJ (2007) The AAA+ protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. J Cell Biol 176 (2):141–146

Hoffmann M, Bellance N, Rossignol R, Koopman WJ, Willems PH, Mayatepek E, Bossinger O, Distelmaier F (2009) C. elegans ATAD-3 is essential for mitochondrial activity and development. PLoS ONE 4(10):e7644

Hubstenberger A, Labourdette G, Baudier J, Rousseau D (2008) ATAD 3A and ATAD 3B are distal 1p-located genes differentially expressed in human glioma cell lines and present in vitro anti-oncogenic. Exp Cell Res 314(15):2870–2883

Kamath RS, Ahringer J (2003) Genome-wide RNAi screening in Caenorhabditis elegans. Methods 30:313–321

Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M et al (2003) Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 421:231–237

Piano F, Schetter AJ, Morton DG, Gunsalus KC, Reinke V, Kim SK, Kemphues KJ (2002) Gene clustering based on RNAi phenotypes of ovary-enriched genes in C. elegans. Curr Biol 12:1959–1964

Schaffrik M, Mack B, Matthias C, Rauch J, Gires O (2006) Molecular characterization of the tumor-associated antigen AAA-TOB3. Cell Mol Life Sci 63(18):2162–2174

Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV, Kamath RS, Fraser AG, Ahringer J, Plasterk RH (2003) Genome-wide RNAi of C. elegans using the hypersensitive rrf-3 strain reveals novel gene functions. PLoS Biol 1:E12

Sonnichsen B, Koski LB, Walsh A, Marschall P, Neumann B, Brehm M, Alleaume AM, Artelt J, Bettencourt P, Cassin E et al (2005) Full-genome RNAi profiling of early embryogenesis in Caenorhabditis elegans. Nature 434:462–469

Wang Y, Bogenhagen DF (2006) Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane. J Biol Chem 281 (35):25791–25802

Zeller KI, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV (2003) An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. Genome Biol 4(10):R69