Biochimica et Biophysica Acta 1853 (2015) 3165–3181

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Nucleic acid import into mitochondria: New insights into the translocation pathways

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ARTICLE INFO

Article history:
Received 29 May 2015
Received in revised form 16 August 2015
Accepted 10 September 2015
Available online 12 September 2015

Keywords:
Import factor
Mitochondria
Nucleic acid transport
Plant
Yeast
Permeability transition pore

ABSTRACT

Mitochondria have retained indispensable but limited genetic information and they import both proteins and nucleic acids from the cytosol. RNA import is essential for gene expression and regulation, whereas competence for DNA uptake is likely to contribute to organellar genome dynamics and evolution. Contrary to protein import mechanisms, the way nucleic acids cross the mitochondrial membranes remains poorly understood. Using proteomic, genetic and biochemical approaches with both plant and yeast organelles, we develop here a model for DNA uptake into mitochondria. The first step includes the voltage-dependent anion channel and an outer membrane-located precursor fraction of a protein normally located in the inner membrane. To proceed, the DNA is then potentially recruited in the intermembrane space by an accessible subunit of one of the respiratory chain complexes. Final translocation through the inner membrane remains the most versatile but points to the components considered to make the mitochondrial permeability transition pore. Depending on the size, DNA and RNA cooperate or compete for mitochondrial uptake, which shows that they share import mechanisms. On the other hand, our results imply the existence of more than one route for nucleic acid translocation into mitochondria.

1. Introduction

Mitochondria have retained their own genetic system but in many organisms their genome does not encode a complete set of transfer RNAs (tRNAs), so that they take up nuclear-encoded tRNAs from the cytosol. Originally proposed in the 1960s for *Tetrahymena pyriformis* [1], this biological process has been progressively extended to most eukaryotic lineages [2–4]. The imported species account for one up to all mitochondrial tRNAs, depending on the organism. On the other hand, mitochondria in mammalian cells take up nuclear-encoded 5S ribosomal RNA (rRNA) [5] and RNase P/RNase MRP RNA [6], although the role of such RNAs in the organelles is still a matter of debate. Moreover, recent studies in mammals implied the outsourcing of specific nuclear-encoded microRNAs (miRNAs) into the mitochondria, presumably for specific regulation pathways ([7,8], reviewed in [9]). Mitochondria also take up DNA. The seminal studies with plant mitochondria [10] were extended to mammalian [11] and *Saccharomyces cerevisiae* [12] organelles. Exogenous DNA imported into isolated mitochondria functionally reaches the nucleoids and joins the organelle DNA metabolism, as it can be transcribed [10,11] or repaired [13,14] *in organello*. Appropriate constructs can also recombine with the resident mtDNA [15]. DNA trafficking might account for paternal inheritance of specific mitochondrial plasmids in plants [16] and contribute to genetic fluxes, organelle DNA maintenance and expansion, or pathogenic DNA-induced mitoptosis [10,17].

The data altogether imply that, as the uptake of nuclear-encoded proteins, trafficking of nucleic acids is a major mitochondrial process. Deciphering the underlying mechanisms is a difficult task, as these defy the common view on membrane properties. The answers are expected to highlight unprecedented functionalities and open new ways to master the mitochondrial genetic system. Nevertheless, the challenge of understanding which channel(s) and which driving force(s) can mediate the translocation of nucleic acids of over 11 kb [16] into mitochondria remains mostly intact. The mechanisms underlying organellar uptake of specific RNAs have been the subject of extensive and sometimes controversial research [4,18,19]. We proposed earlier the yeast *S. cerevisiae* as a powerful genetic model to investigate the protein partners involved in mitochondrial DNA uptake [12]. Together with relevant biochemical data, the strategy enabled to establish that the ß-barrel integral membrane protein VDAC (voltage-dependent anion channel) is a major player in DNA translocation through the mitochondrial outer membrane [12]. The VDAC is also required for tRNA uptake into plant [20] but not trypanosomatid [21] organelles. Direct data on nucleic acid translocation through the tight and polarized organellar inner membrane are scarce [2–4,22]. The regular pre-protein

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http://dx.doi.org/10.1016/j.bbamcr.2015.09.011
0167-4889/

import complex was proposed to mediate uptake of cytosolic tRNA-^{Lys}(CUU) into yeast mitochondria [23] and 5S rRNA into human organelles [24]. An inner membrane "RNA import complex" (RIC) was isolated from *Leishmania tropica* mitochondria [25] but remained a subject of controversy [4,26] and raised an editorial expression of concern [27]. In mammalian mitochondria, the polynucleotide phosphorylase (PNPASE) enzyme may function as an RNA receptor in the intermembrane space [6]. We identified a possible involvement of the adenine nucleotide translocator (ANT) in the uptake of DNA through the mitochondrial inner membrane in plants [10]. As a whole, there has been little progress over several decades of investigation in the understanding of these transport processes.

In the present studies, we used proteomic, biochemical and genetic approaches with both plant and yeast organelles to deepen the investigation of the mechanisms of nucleic acid uptake into mitochondria, taking mainly DNA of different sizes as a substrate. Proteomic approaches based on DNA-mediated masking of mitochondrial proteins identified putative partners of the VDAC in the transport through the mitochondrial outer membrane. Their functional involvement was confirmed by genetic analysis. Moreover, the strategy revealed a novel import factor likely to recruit the substrate in the intermembrane space of plant mitochondria. Further special attention was given to the members of the mitochondrial carrier family [28,29] as potential mediators of nucleic acid translocation across the inner membrane. A differential functional involvement of nucleotide carriers in DNA import was established between plant and yeast mitochondria. Further assays with *S. cerevisiae* organelles highlighted the importance of membrane proteins that control mitochondrial dynamics and organization. Introduction of tRNA transcripts into DNA import assays pointed to the interplay between DNA and RNA transport. On the other hand, our results imply that several alternative pathways co-exist in a given organism for nucleic acid transport into mitochondria, especially to account for the import of small or large nucleic acids.

2. Materials and methods

2.1. S. cerevisiae strains

The W303 [30], JLY-73 [31] and JL-1-3 [32] strains were provided by L. Sabova (Cancer Research Institute, Bratislava, Slovakia). The Δ*mir1* and Δ*pic2* single mutants, as well as the Δ*mir1*/Δ*pic2* double mutant [33] were provided by G. Dujardin (Centre de Génétique Moléculaire, Gif-sur-Yvette, France). The WΔM strain [24] was provided by I. Tarassov (CNRS–University of Strasbourg, France). The Y10000, Y17227, Y11398, Y10246, Y23332, Y13896, Y11458, Y14229, Y15606, Y13370 and Y17559 strains were purchased from the Euroscarf collection (Institute of Molecular Biosciences, Johann Wolfgang Goethe-University, Frankfurt, Germany) [34]. *S. cerevisiae* strains were usually grown aerobically in 2% (w/v) galactose medium.

2.2. Arabidopsis thaliana cell culture

A. thaliana cell suspensions, ecotype Columbia, line T87-C33 [35] were dark grown at 24 °C in Gamborg B5 medium (Duchefa) supplemented with 2% (w/v) sucrose and 1 mg/L 2.4 D, final pH 5.7. Cultures were transferred to fresh medium every week and harvested 5 days after transfer when preparing mitochondria.

2.3. A. thaliana mutant line

The *A. thaliana* mutant line GABI_753D05 carrying a T-DNA insertion in the copper ion binding protein gene At2g27730 in the ecotype Columbia Col-0 background was obtained from the Nottingham Arabidopsis Stock Centre (UK). The T-DNA insertion was identified by PCR using the gene- and T-DNA-specific primers AtCBP_1 (5′-CTTAGC GAAGAGGAGAGAGCC-3′), AtCBP_2 (5′-TTCCAAAGTGTCTCACAAGGG-3′) and P-787 (5′-CGCCTATAAATACGACGCATCGTA-3′). The insertion was shown to be composed of at least two T-DNA copies head to head. Homozygous plants were selected and self-propagated. Wild-type Col-0 and mutant *A. thaliana* plants were grown on soil at 23 °C under a 16 h light/8 h dark photoperiod. For *in vitro* cultures, surface-sterilized seeds were sown on agar plates containing MS255 (Duchefa) supplemented with 1% (w/v) sucrose and stratified at 4 °C for 3 days to synchronize germination. Plants were subsequently grown under 16 h light/21 °C and 8 h dark/17 °C conditions. Knockout of the gene was confirmed by RT-qPCR run with RNA samples isolated from five-week-old plants. Total RNA was extracted with TRI-Reagent (MRC), treated with RNase-free DNase (Promega) and reverse transcribed with GoScript™ RT (Promega). qPCR was performed in a LightCycler480, with the SYBR Green I Master Mix (Roche). *ACTIN2* (At3g18780) and *GAPDH* (At1g13440) were taken as reference genes.

2.4. Isolation of mitochondria

Yeast cells were grown at 30 °C in YPGal medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v galactose, pH 5.5) and mitochondria were isolated according to Daum et al. [36] and Weber-Lotfi et al. [12]. For proteomic analyses, yeast mitochondria were further purified as described by Meisinger et al. [37].

Plant mitochondria were isolated from potato (*Solanum tuberosum*) tubers or from cauliflower (*Brassica oleracea*) following previously described methods based on continuous Percoll gradients [10,38]. For proteomic experiments, *A. thaliana* mitochondria were purified from cell suspensions using discontinuous Percoll gradients as described by Sakamoto et al. [39]. For mutant studies, mitochondria were extracted from *A. thaliana* seedlings grown *in vitro* on agar plates and harvested at a four-leaf stage. In that case, organelles were isolated essentially according to protocols reported by Day et al. [40] and Sweetlove et al. [41].

Total protein content of final mitochondrial suspensions was estimated using the Bio-Rad DC Protein Assay. When comparing mitochondria from different strains, protein amounts to be introduced into import assays were further fine tuned on the basis of the VDAC content. For this, western blots were prepared, probed with anti-VDAC antibodies and revealed on film with the ECL chemiluminescence kit (GE Healthcare). Films were scanned and signals quantified with the Image Gauge software. If needed, protein amounts were readjusted and verified again on a second western blot.

2.5. Respiration assays

For respiration assays, oxygen consumption of isolated mitochondria was measured in an oxygraph chamber (Hansatech) using 10 mM succinate as a substrate in previously described conditions [38,42]. Respiration control was calculated as the ratio between the oxygen consumption rate of the mitochondria upon addition of 200 μM ADP ("active state" or State III) and the rate in the absence of added ADP or when the added ADP was entirely converted to ATP ("resting state" or State IV).

2.6. Blue native PAGE and respiratory complex activity

Blue native-PAGE was carried out according to protocols described by Jänsch et al. [43] and Meyer et al. [44]. *A. thaliana* mitochondrial membranes were solubilized for 20 min on ice with 1.5% (w/v) n-dodecylmaltoside in ACA buffer (750 mM amino dicaproic acid, 50 mM Bis–Tris pH 7.0, 0.5 mM EDTA). *S. cerevisiae* mitochondrial complexes were solubilized with 5% (w/v) digitonin in ACA buffer. Insoluble material was eliminated by centrifugation at 105,000 *g* for 15 min and the supernatant was supplemented with Serva Blue G at a final concentration of 0.25% (w/v) before loading on gel. After running for 5 h, the cathode compartment was filled with buffer deprived of