Splicing before import – An intein in a mitochondrially targeted preprotein folds and is catalytically active in the cytoplasm in vivo

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Received 17 April 2000; received in revised form 8 June 2000

Edited by Gunnar von Heijne

Abstract Nuclear-encoded mitochondrial proteins are cytoplasmically synthesized and imported into the organelle. The intein-containing RecA protein of *Mycobacterium tuberculosis*, with or without the CoxIVp mitochondrial targeting signal (MTS), was used to determine where a protein targeted to mitochondria folds and becomes catalytically active. Analysis of fractions from *Saccharomyces cerevisiae* cells expressing RecA without the MTS revealed that RecA and intein proteins remained cytoplasmic. With the MTS, most of RecA was directed to mitochondria, while most of the intein remained in the cytoplasm. The intein therefore folds into a catalytically active state in the cytoplasm prior to RecA import into mitochondria.

Key words: Mitochondrial import; Protein splicing; *Saccharomyces cerevisiae*

1. Introduction

Hundreds of proteins are necessary for mitochondrial function, yet few are coded inside the organelle. The majority of mitochondrial proteins are translated in the cytoplasm and then transported into the organelle [1]. Mitochondrial preproteins are recognized by components of the TOM (translocase of the outer mitochondrial membrane) complex, generally through an N-terminal mitochondrial targeting sequence (MTS) [2]. Preproteins must be in an unfolded or loosely folded state for translocation into the organelle [3]. One way that a preprotein could be imported in an unfolded state is through a cotranslational mechanism, in which a polypeptide chain would be translocated directly into the organelle as it is being synthesized [4–6]. However, a requirement for cotranslational import into mitochondria has been difficult to support since post-translational import occurs efficiently [1]. Post-translational import implies that there must be mechanisms for preproteins to achieve or maintain an import-competent state after synthesis. Various cytoplasmic proteins including mitochondrial import stimulation factor [7], heat shock protein 70 (hsp70) [8], and the DnaJ homologue Ydj1p [9] have limited roles in import, and presumably prevent folding of precursors until they are engaged by the mitochondrial import machinery. Recently, mitochondrial hsp70 has been shown to unfold a translocation intermediate at the mitochondrial surface by interacting with its MTS in the matrix [10,11]. This observation suggests that proteins may be capable of folding into their native structures prior to unfolding at the mitochondrial surface.

Using a fusion protein where an intein-containing passenger is fused downstream of an MTS, we determined the intracellular location where the intein folds into a catalytically active protein. Inteins are proteins that can splice themselves from another protein. Protein splicing involves the post-translational excision of an internal region (the intein) from a precursor protein with the subsequent ligation of the remaining external protein fragments (exteins) [12,13]. Splicing occurs once the intein-containing precursor protein folds into a catalytically active conformation. All of the information necessary for splicing is present in the intein sequence plus the first amino acid of the carboxy-terminal extein, which must be a Cys, Ser, or Thr. No additional proteins are necessary. Thus, the intracellular location of the intein indicates where in the cell the precursor protein folds into a catalytically active, splicing-competent conformation. We show that the majority of the intein in a mitochondrially targeted preprotein is in the cytoplasm. These data demonstrate that the intein folds and splices in the cytoplasm before the remaining preprotein is imported into the organelle.

2. Materials and methods

2.1. Plasmid construction

Plasmid pEJ134 contains the *recA* gene of *Mycobacterium tuberculosis* [14]. To facilitate subcloning of the *recA* gene, an *Eco*RV site at the 5′-end and a *Cla*I site near the 3′-end of the *recA* open reading frame (ORF) were inserted by site-directed mutagenesis [15]. The *Eco*RV site was inserted directly following the ATG codon using the antisense primer 5′-TCCCGATCGGCGGTCTGCGTCA[GATATC]CATGGTGCCTCTCCTGTGGT-3′. The *recA* sequence is underlined with the restriction site bracketed. Two extra bases (CA) not found in the *recA* gene were added to place the cytochrome oxidase subunit IV (COXIV) leader sequence in frame with the *recA* ORF in the final construct. The *Cla*I site was inserted downstream of the stop codon using the antisense primer 5′-GCGGGTGCGGGATCGCGCGGCC[ATCGAT]TGAGCAGGCGCAGGCACAG-3′. The resulting 2.4 kb *Eco*RV–*Cla*I fragment containing the RecA protein was fused in frame with the first 22 amino acids of the *Saccharomyces cerevisiae* CoxIVp MTS (MLSLRQSIRFFLPATRTLCSSR) [16]. An *Eco*RI–*Cla*I fragment containing the CoxIVp MTS fused to the *recA* gene ($RecA_{mito}$) was cloned into BSIIKS-. The sequence between the *Eco*RI site and the start codon (underlined) of the CoxIVp MTS is 5′-GAATTCCATACAAATAGATAACAAGCACAATG-3′. The CoxIVp MTS was removed from the $RecA_{mito}$ construct by site-directed mutagenesis using the antisense primer 5′-TCCCGATCGG-

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0014-5793/00/$20.00
PII: S0014-5793(00)01736-1

CGGTCTGCGTCAT[TGTGCTTGTTATCTATTTG]-3′ to create the control with the same upstream sequence but lacking the MTS. The *recA* sequence is underlined and upstream sequence is enclosed in brackets. Successful removal of the targeting sequence was confirmed by the loss of a *Xba*I site present in the MTS. For expression in yeast, the two constructs were cloned behind the ADH1 promoter in the plasmid G18 [17] as *Eco*RI–*Cla*I fragments, forming G18/RecA and G18/RecA$_{mito}$.

2.2. Cell fractionation

The proteinase-deficient *S. cerevisiae* strain BJ5457 (MATα, *ura*3–52, *trp*1, *lys*2–801, *leu*2Δ1, *his*3Δ200, *pep4*::HIS3, *prb*1Δ1.6R, *can*1, GAL) (American Type Culture Collection, number 208282) was used to minimize protein degradation. BJ5457(G18), BJ5457(G18/RecA), and BJ5457(G18/RecA$_{mito}$) were grown in SCglu-ura (0.67% yeast nitrogen base without amino acids supplemented with appropriate amino acids and 2% glucose) or in GE (3% glycerol, 2% ethanol, 2% peptone, 1% yeast extract) medium to mid-exponential phase. Cell fractionations were performed basically as described by Wolfe et al. [18].

2.3. SDS–PAGE and immunoblot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the Laemmli system [19]. Protein concentrations in the cellular fractions were determined using the Bio-Rad *DC* Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. One hundred micrograms of total cell lysate proteins were separated in a 10% polyacrylamide gel. To enable comparisons between the fractions and the total cell lysate, amounts of the PMS and mitochondrial fractions equivalent to the corresponding total cell lysate were also loaded on the gel. Proteins were transferred to Immobilon-P PVDF membranes (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions using a Hoefer Transphor Electrophoresis Unit at 100 mA constant current for 16 h at 4EC. Membranes were probed with antibodies against different proteins which were then detected by chemiluminescence using an ECL or ECL Plus kit (Amersham, Arlington Heights, IL, USA), according to the manufacturer's instructions. The anti-intein antibody [20] and anti-RecA antibody were used at a 1:10 000 dilution and 1:1000 dilution, respectively. The anti-F1β-ATPase antibody used as a mitochondrial marker was obtained from Dr. Michael Douglas and used at a 1:10 000 dilution. The anti-phosphoglycerate kinase (PGK) antibody used as a cytoplasmic marker was purchased from Molecular Probes (Eugene, OR, USA) and used at a 1:125 000 dilution. Images were analyzed using ImageQuant® software (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. Addition of an MTS to the RecA protein does not inhibit intein splicing

The passenger protein used in this study was the intein-containing RecA protein of *M. tuberculosis*. The *recA* gene codes for a 440 amino acid intein located between amino acids 254 and 255 of the 350 amino acid RecA protein [14,20]. The gene codes for an 85-kDa precursor that undergoes protein splicing to produce the 47-kDa intein and the 38-kDa RecA protein. The DNA encoding the 85-kDa precursor was placed downstream of the DNA coding for the nuclear-encoded cytochrome oxidase subunit IV MTS, known to direct heterologous proteins into mitochondria [4,5,16]. Analysis of products synthesized in vitro from a coupled transcription/translation system programmed with BSIIKS-/RecA revealed two proteins with apparent masses of 38 kDa and 47 kDa (Fig. 1, lane RecA). The 38-kDa product was the expected size for the RecA protein, while the 47-kDa product corresponded to the 440 amino acid intein. The coupled system programmed with BSIIKS-/RecA$_{mito}$ also yielded two products, the intein and the 42-kDa RecA–CoxIVp–MTS fusion. Surprisingly, the autoradiographic signal for RecA, which

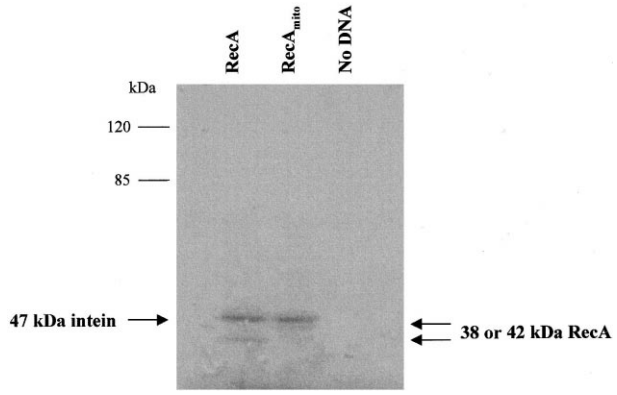

Fig. 1. Addition of an MTS to the RecA protein does not inhibit intein splicing. The two constructs BSIIKS-/RecA and BSIIKS-/RecA$_{mito}$ were expressed from the T7 promoter and products labeled with ^{35}S-methionine using the TNT® T7 Coupled Reticulocyte Lysate System from Promega (Madison, WI, USA) according to the manufacturer's instructions. Products were separated by SDS–PAGE using a 7.5% polyacrylamide gel. The gel was fixed for 30 min in 25% methanol/7% acetic acid, rinsed in distilled H_2O, and treated with Enhance (NEN, Boston, MA, USA) for 1 h at room temperature. The gel was then dried and exposed to film. Proteins produced from BSIIKS-/RecA include the 47-kDa intein and 38-kDa RecA protein (lane labeled RecA). Proteins produced from BSIIKS-/RecA$_{mito}$ include the 47-kDa intein and the 42-kDa RecA–CoxIVp–MTS fusion protein (lane labeled RecA$_{mito}$). No products are visible in the negative control lane with no DNA (lane labeled No DNA).

contains nine methionine residues, was less than that of the intein, which contains six methionine residues. Finding the RecA signal decreased suggested that the RecA protein was less stable than the intein protein in this in vitro system. No unspliced precursors were observed with either construct, indicating that splicing was efficient. Thus, placing an MTS at the amino-terminus of the RecA/intein precursor did not disrupt splicing.

3.2. RecA precursor is expressed and spliced in yeast

To determine whether the mitochondrially targeted, intein-containing protein could be expressed and spliced in yeast, the genes coding for these proteins were cloned into the yeast expression vector G18, downstream of the *ADH1* promoter [21]. Total protein extracts from BJ5457(G18) and BJ5457(G18/RecA$_{mito}$), grown on medium containing either glucose or glycerol, were subjected to Western analysis with polyclonal anti-intein antibodies. A signal corresponding to the 47-kDa intein was observed in extracts from cells containing G18/RecA$_{mito}$ but not from cells containing G18 alone (data not shown, see Figs. 2 and 3). Therefore, the bacterial RecA precursor was expressed and spliced in yeast cells grown in fermentable and non-fermentable media.

3.3. RecA, but not the intein, is mitochondrial in cells growing by fermentation

If the intein-containing MTS preprotein folds in the cytoplasm, then the intein should be located in the cytoplasm. Alternatively, if the protein does not fold in the cytoplasm prior to mitochondrial import, then the intein should be located in mitochondria. To determine the location of the intein and RecA proteins, cellular fractions from cells grown in glucose were blotted and probed with anti-intein, anti-RecA, anti-F1β-ATPase and anti-PGK antibodies. Membranes

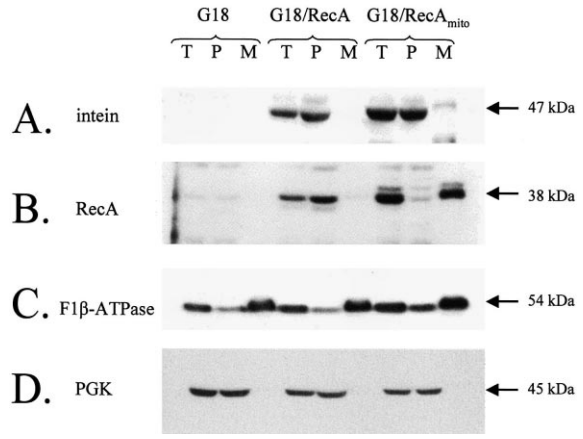

Fig. 2. RecA, but not the intein, is mitochondrial in cells growing by fermentation. BJ5457(G18), BJ5457(G18/RecA), and BJ5457(G18/RecA$_{mito}$) cells were grown in SCglu-ura medium to mid-exponential phase and fractionated into total (T), post-mitochondrial supernatant (P, cytoplasmic proteins), and mitochondrial (M) fractions. The PVDF membrane was probed sequentially with anti-intein (A), anti-RecA (B), anti-F1β-ATPase (C), and anti-PGK (D) antibodies.

were not stripped between antibody reactions to minimize the potential loss of antigens. Analysis of the subcellular location of the intein using the polyclonal anti-intein antibodies revealed that, without the MTS, all of the 47-kDa intein was in the post-mitochondrial supernatant (PMS) (Fig. 2A, G18/RecA lane P). A small amount of the intein was found in the mitochondria from cells containing G18/RecA$_{mito}$ (Fig. 2A, G18/RecA$_{mito}$ lane M), but most of the intein was located in the PMS (Fig. 2A, G18/RecA$_{mito}$ lane P). The different mobility of the intein in the mitochondrial lanes is reproducible and probably due to the difference in the amount of protein in total and PMS fractions relative to mitochondrial fractions. No intein signal was detected in any fraction from the control cells transformed with vector alone (Fig. 2A, G18 lanes). Thus, most of the preprotein adopted a splicing-competent, catalytically active conformation in the cytoplasm.

In contrast to the results obtained for the intein, the majority of the 38-kDa RecA protein in cells containing G18/RecA$_{mito}$ was located in mitochondria (Fig. 2B, G18/RecA$_{mito}$ lane M). The two forms of RecA observed in these cells (Fig. 2B, G18/RecA$_{mito}$ lane T) probably represent the RecA protein with and without the CoxIVp MTS, which contains a site recognized by the mitochondrial processing peptidase [16]. The shorter form of the two RecA proteins was the same size as RecA lacking the MTS, which was found exclusively in the cytoplasm (Fig. 2B, G18/RecA lane P). The small amount of processed RecA in the PMS fraction from G18/RecA$_{mito}$ cells (Fig. 2B, G18/RecA$_{mito}$ lane P) appeared to result from mitochondrial lysis during fractionation since a small amount of the β-subunit of the F1-ATPase, used as a mitochondrial marker, was also observed in the PMS fraction (Fig. 2C, lanes P). The cytoplasmic marker PGK showed that the mitochondrial fractions contained little cytoplasmic contamination (Fig. 2D). The location of intein and RecA derived from the MTS-containing precursor revealed that, while the RecA was directed to and imported into mitochondria, the majority of the intein remained in the cytoplasm. Thus, the majority of the precursor folded into a catalytically active, splicing-competent conformation before the RecA was imported into mitochondria.

3.4. The intein-containing RecA precursor folds into a catalytically active state in the cytoplasm of cells growing by respiration

The previous experiment showing that the RecA precursor protein folded into a splicing-competent state in the cytoplasm was performed with cells harvested during exponential growth in glucose. Since growth of yeast on a non-fermentable carbon source such as glycerol will enhance mitochondrial biogenesis, we asked whether changing carbon source from glucose to glycerol would alter the cellular location where the intein-containing precursor folds into a catalytically active conformation. Cell fractionations were performed on the same strains used in Fig. 2, except that cells were harvested during exponential growth in medium containing glycerol as the carbon source. In cells where the RecA precursor contained the CoxIVp MTS, a small amount of the intein was located in the mitochondria (Fig. 3A, G18/RecA$_{mito}$ lane M), with most of the intein protein in the cytoplasm (Fig. 3A, G18/RecA$_{mito}$ lane P). Under these conditions, the vast majority of the RecA protein in total cell extracts (Fig. 3B, G18/RecA$_{mito}$ lane T) was in the processed state (38 kDa), indicating efficient import. The unprocessed form (42 kDa) was visible in the cytoplasm (Fig. 3B, G18/RecA$_{mito}$ lane P). The recovery of processed RecA protein in the mitochondrial fraction was low. This result did not stem from inefficient recovery of mitochondria since the β-subunit of the F1-ATPase was efficiently recovered (Fig. 3C). Furthermore, the marker PGK was also efficiently recovered in the cytoplasm (Fig. 3D). Consequently, the selective loss of the bacterial RecA is related to its relative instability in mitochondria. In contrast to the RecA protein, the intein was both efficiently recovered and located predominantly in the PMS (Fig. 3A, G18/RecA$_{mito}$ lane P). Therefore, even under glycerol growth conditions the majority of the intein-containing RecA precursor adopted a catalytically active conformation in the cytoplasm.

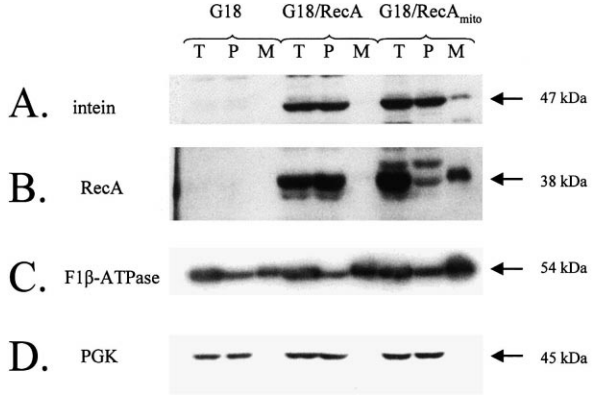

Fig. 3. The intein-containing RecA precursor folds into a catalytically active state in the cytoplasm of cells growing by respiration. The yeast cells were grown to mid-exponential phase in glycerol medium to increase mitochondrial function and fractionated into total (T), post-mitochondrial supernatant (P, cytoplasmic proteins), and mitochondrial (M) fractions. The PVDF membrane was probed sequentially with anti-intein (A), anti-RecA (B), anti-F1β-ATPase (C), and anti-PGK (D) antibodies.

4. Discussion

The data reported here show that most of the intein in a mitochondrially targeted, intein-containing preprotein folds into a catalytically active, splicing-competent conformation in the cytoplasm prior to import of the remaining preprotein into the organelle. Although there are no means to determine the folding state of RecA directly, our results support a model in which a preprotein is capable of folding into a catalytically active state within the cytoplasm prior to being unfolded at the mitochondrial surface for subsequent import. These data independently support earlier studies demonstrating the post-translational import of mitochondrial proteins [22,23]. Furthermore, these data are consistent with recent in vitro observations that a precursor can be actively unfolded on the mitochondrial surface [10,11], and indicate that in vivo a mitochondrial precursor can reach an import-competent state once it has been folded into a catalytically active state in the cytoplasm.

These data are not consistent with most of the protein traversing the mitochondrial membrane by a cotranslational mode of import or with the extensive involvement of cytosolic chaperones in maintaining this mitochondrial preprotein in an unfolded, import-competent state. While it is possible that the precursor could adopt a catalytically active conformation as it is being synthesized and concurrently imported, a consideration of certain parameters measured for the import pathway and the size of the RecA protein make this possibility unlikely. The intein itself is 440 amino acids long; both amino- and carboxyl-termini must be present in the same intracellular compartment for splicing to occur. Since the rate of peptide chain elongation is two–four amino acids per s [1], cotranslational mitochondrial import would have to be slower than the approximately 1.5 min required to synthesize both ends of the intein on the cytoplasmic side of the organelle. Moreover, additional time is necessary to synthesize the 254 amino acid amino-terminal extein of RecA located between the MTS of the preprotein and the intein. Thus, cotranslational import would require that a single intein-containing precursor occupies a TOM complex for at least 3 min. Estimates of the mass of mitochondrial protein, relative to the number of translocase complexes, indicate that such a slow rate of import would not maintain mitochondrial mass given the rapid doubling time of most yeast cells [1].

Folding of the precursor into a splicing-competent form prior to import into mitochondria also argues against the extensive involvement of cytosolic chaperones in maintaining this preprotein in an import-competent state as it transits through the cytoplasm. Some mitochondrial import models propose that presequences are recognized early, as they exit from the ribosome, by factors that target preproteins to the organelle [1,24–26]. Part of this targeting is the recruitment of cytoplasmic chaperones to the preprotein to maintain it in an import-competent state prior to import. While our data argue against this model for the RecA precursor as a whole, the possibility exists that the intein and RecA portions of the precursor fold at different rates and thus are capable of interacting differently with cytosolic chaperones. For example, the intein could fold at a faster rate than RecA, achieving a splicing-competent conformation while evading interactions with cytosolic chaperones; whereas the slower folding RecA could interact with the chaperones and be maintained in an import-competent state until translocation into the organelle. This hypothesis is inconsistent with models in which the mitochondrial targeting signal exclusively targets the remainder of the precursor to a pathway where it is maintained in an import-competent state prior to translocation into the organelle. Our data show that the entire intein-containing precursor is not maintained in an unfolded conformation during transport through the cytoplasm.

The finding that the majority of the intein-containing precursor is imported post-translationally does not mean that other minor mechanisms are not involved. In fact, the small amount of intein found in the mitochondria could be imported via cotranslational import, or cytosolic chaperones may prevent a small portion of the precursor from folding until import is complete. Having multiple pathways and mechanisms involved in mitochondrial import may provide flexibility which could be important for processing a range of precursors with distinct properties. Perhaps alternate pathways provide mechanisms to enable the import process to respond to various environmental conditions. The assay system described here has the potential to discriminate among these multiple mechanisms and assess their relative contribution to the import of different precursors. This system also may be used to measure the effects of alterations in the mitochondrial import apparatus brought about by mutations or other genetic manipulations. Furthermore, this approach could be used to examine protein targeting into other organelles such as the endoplasmic reticulum or vacuoles.

Acknowledgements: This work was supported by a grant from the National Science Foundation to A.K.H. and N.C.M. We thank Dr. Tom Fox for helpful discussions about using inteins to examine mitochondrial import. We thank Dr. Faye Austin and Marlene C. Steffen for many helpful discussions and for critical reading of the manuscript.

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