Translation-coupled Translocation of Yeast Fumarase into Mitochondria in Vivo*

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Fumarase represents proteins that cannot be imported into mitochondria after the termination of translation (post-translationally). Utilizing mitochondrial and cytosolic versions of the tobacco etch virus (TEV) protease, we show that mitochondrially targeted fumarase harboring a TEV protease recognition sequence is efficiently cleaved by the mitochondrial but not by the cytosolic TEV protease. Nonetheless, fumarase was readily cleaved by cytosolic TEV when its import into mitochondria was slowed down by either (i) disrupting the activity of the TOM complex, (ii) lowering the growth temperature, or (iii) reducing the inner membrane electrochemical potential. Accessibility of the fumarase nascent chain to TEV protease under such conditions was prevented by low cycloheximide concentrations, which impede translation. In addition, depletion of the ribosome-associated nascent polypeptide-associated complex (NAC) reduced the fumarase rate of translocation into mitochondria and exposed it to TEV cleavage in the cytosol. These results indicate that cytosolic exposure of the fumarase nascent chain depends on both translocation and translation rates, allowing us to discuss the possibility that import of fumarase into mitochondria occurs while the ribosome is still attached to the nascent chain.

The majority of mitochondrial proteins are encoded in the nucleus, translated on cytosolic ribosomes, and imported into mitochondria. Most matrix-targeted proteins are synthesized as precursors carrying an N-terminal mitochondrial targeting sequence (MTS),2 which is usually cleaved off by the mitochondrial processing peptidase (MPP) upon entry into the matrix. Precursor proteins are recognized by receptors on the mitochondrial surface and are translocated across the outer membrane through the TOM complex. The precursors are then translocated across the inner membrane through the TIM23 complex in a membrane potential-dependent manner (1, 2). According to in vivo and in vitro studies, most mitochondrial proteins can be successfully imported into mitochondria post-translationally (3, 4). Nevertheless, there are indications for the existence of co-translational or translation-coupled import of proteins into mitochondria. For example, under normal growth conditions (in vivo), mitochondrial precursor proteins are essentially not detected in the cytosol even in extremely short pulse labeling experiments (5). Moreover, cycloheximide-treated yeast cells accumulate a large number of cytosolic polypeptides on the surface of mitochondria, and these polypeptides are enriched with mRNA encoding mitochondrial proteins (6–8). These experiments suggest that import into mitochondria may occur co-translationally under normal physiological conditions; however, for most mitochondrial proteins, co-translational import is not obligatory.

Fumarase (9, 10) represents a small group of proteins, such as manganese-dependent superoxide dismutase 2 (Sod2p) (11) and the major adenylate kinase (Aky2) (12, 13), which cannot be imported into mitochondria after the termination of their translation. In vivo, precursors of these mitochondrial proteins, which were forced to accumulate in the cytosol, could not be chased into mitochondria (10, 13). In addition, in vitro, precursors synthesized to completion in reticulocyte lysate could not be imported into isolated mitochondria (9, 12).

We have recently shown that the MTS of fumarase can be cleaved by externally added mitochondrial processing peptidase en route to mitochondria in an in vitro translation-translocation coupled reaction (14). In the current study, we have expressed the tobacco etch virus (TEV) protease (15, 16) in the cytosol or in mitochondria of yeast and asked whether fumarase harboring the protease recognition site can be cleaved by the cytosolic protease during import in vivo. Additionally, the mitochondrially targeted TEV protease enables us to establish an import kinetics assay to determine in vivo the translation coupled import time frame. These experimental systems allowed us to investigate the kinetics of mitochondrial protein import in vivo and the possible relationship between the translation and translocation systems.

EXPERIMENTAL PROCEDURES

Strains—The Saccharomyces cerevisiae strains used were YPH499 (MATa ade2-101, lys2-801, ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1), MH272-3fa (EGD wild type) (MATa, ura3, leu2, his3, ade2, trp1), YRLG16 (egd1Δ, egd2Δ) (MATa leu2, trp1, his3, ura3, ade2; egd2::ADE2, egd1::URA3) (28) KKY1 (TOM40 wild type) (MATa/a his3-Δ200/his3-Δ200 isp42::HIS3/ISP42, leu2-3,112/leu2-3, 112 lys2-801/LYS2, ade2-101/ADE2, suc2-Δ9/suc2-Δ9, trp1-Δ901/trp1-Δ901 ura3-52/ura3-52), KKY3 (tom40 ts) (MATa, his3-Δ200, isp42::HIS3,
Plasmids—The plasmid pYeF1-TEV (17) was kindly provided by Marc Bonneu. Plasmid pYEBG713, kindly provided by the group of Walter Neupert, encodes the TEV protease preceded by the Su9-ATPase MTS (79 amino acids). The TEV fragment, in frame with this Su9 presequence, was cloned into the yeast expression vector pYeDP1 and resulted in plasmid pYEBG713 encoding the fusion protein Su9-(1–79)-TEV. Plasmids pFum92TVS and pFum488TVS6H are described in this study. To create pFum92TVS, the TEV site was amplified using primers SeqYep (5’-attagcagtaagctgca-3’) and TEVBglII (5’-agatctagatgaaatacaattttctggatgccatcctccccc-3’) and pFT2 (10) as template. The resulting product was cut with SalI and BglII and cloned into pFT2. pFum488TVS6H was constructed using a two-step PCR procedure. In the first step, primers DraIIIF 5’-gatctagatgaaataacaattttctggatgccatc-3‘ and Fum488TVSR1 5’-ttt agc agc agc ttc ttg agc agc agc ttc ttt agc agc agc ttc ttt agc agc agc ttc ttg cat g-3‘ were used with pFT2 as template. In the second step, primers DraIIIF and primers SeqYep (5’-attagcagtaagctgca-3’) and TEVBglII (5’-agatctagatgaaatacaattttctggatgccatcctccccc-3’) and DraIII and Apal and cloned into pFT2.

Growth Conditions—Strains harboring the appropriate plasmids were grown in synthetic depleted medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose or galactose, CSM dropout mix (QiBiogene) and supplemented with the appropriate amino acids (50 μg/ml).

Metabolic Labeling and Immunoprecipitation—Yeast cultures were grown in synthetic depleted medium containing galactose to an OD600nm of 1.5. Cells were harvested, washed, and resuspended in fresh galactose synthetic depleted medium lacking methionine. Cultures were labeled with 100 μCi of [35S]methionine/cysteine and incubated for the time indicated in the figure legends. Labeling was stopped by the addition of 10 mM sodium azide. When needed, 20 μM CCCP was included in the reaction mixture to disrupt the mitochondrial membrane potential, whereas 40 mM dithiothreitol (DTT) was added to reestablish the membrane potential after CCCP treatment. In pulse-chase experiments, labeling and translation were stopped by the addition of excess “cold” 0.003% methionine, 0.004% cysteine, and 0.001% cycloheximide. Samples were withdrawn at the times indicated and placed on ice, and 10 mM sodium azide was added to the samples. Labeled cells were collected by centrifugation, resuspended in Tris/EDTA buffer, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride, broken with glass beads for 2 min, and centrifuged to obtain the supernatant fraction. Supernatants were denatured by boiling in 1% SDS. Immunoprecipitation was performed in the presence of one-fourth of the total volume of TENN buffer (200 mM Tris, pH 8.0, 20 mM EDTA, 2% Nonidet P-40, 60 mM NaCl), with the appropriate antiserum or monoclonal anti-polyhistidine (Sigma) and protein A magnetic Dynabeads (Dynal Biotech ASA, Oslo, Norway) and then analyzed by SDS-PAGE followed by visualization with the BAS2000 image-analyzing system (Fuji Corp.) and autoradiography.

RESULTS

TEV Protease Can Be Targeted to the Cytosol or to Mitochondria—In order to express the TEV protease in the cytosol or mitochondria, two vectors were used. Plasmid pYEBG713 encodes the TEV protease preceded by the MTS of Su9-ATPase, and plasmid pYeF1-TEV (17) encodes the TEV protease without a preceding MTS. The expression of both TEV constructs is under the GAL10 promoter, which allows regulated expression in galactose-containing medium. To test the cellular location of these TEV proteases, yeast strains expressing either one were induced in galactose medium and were subjected to subcellular fractionation. Probing each fraction with antibodies against Hsp60 or Hxk1, mitochondrial and cytosolic markers, monitored the quality of each fractionation. As seen in Fig. 1, the TEV protease can be targeted exclusively to the cytosol (Fig. 1A, right) or to mitochondria (Fig. 1A, left).

Two plasmids encoding fumarase derivatives were constructed; Fum92TVS encodes fumarase with a TEV recognition site (TVS) at a position corresponding to amino acid 92 of the fumarase protein sequence, and Fum488TVS6H harbors a TEV recognition site inserted at a position corresponding to amino acid 92 of the fumarase protein sequence. Cleavage of this derivative should result in the appearance of a shorter form of the protein. Fum488TVS6H harbors a TEV recognition site at a position corresponding to the last amino acid of fumarase and is followed by a His6 tag. Cleavage of this derivative should cause the elimination of the His6 epitope.

FIGURE 1. Targeted TEV proteases and fumarase-TVS substrates. A, TEV protease can be targeted exclusively to the cytosol or to mitochondria. Yeast cultures induced in galactose medium to express the TEV protease either with or without a mitochondrial targeting sequence were subjected to subcellular fractionation. Equivalent portions from total (T), cytosolic (C), and mitochondrial (M) fractions were analyzed by Western blotting using antisera against the indicated proteins. B, schematic illustration of fumarase derivatives harboring a TEV recognition sites Fum92TVS is fumarase with a TVS inserted at a position corresponding to amino acid 92 of the fumarase protein sequence. Cleavage of this derivative should be performed at the TEV recognition site.

The kinetics of fumarase import, and it allows us to determine the beginning of the process, whereas Fum488TVS6H allows us to investigate its termination. Although the TEV site of the latter is imported post-translationally, it enabled us to test the kinetics of fumarase import, and it allows us to determine
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The import of fumarase derivatives harboring a TEV recognition site cannot occur post-translationally. Yeast cultures induced in galactose medium to express Fum92TVS (lanes 1–4) or Fum488TVS6H (lanes 5–8) were (i) labeled with [35S]methionine/cysteine in the presence of CCCP (lanes 1 and 5), (ii) labeled with [35S]methionine/cysteine in the presence of CCCP (lanes 2 and 6), (iii) labeled with [35S]methionine/cysteine in the presence of CCCP and then chased with excess cold methionine/cysteine in the presence of DTT for an additional 10 min (lanes 3 and 7), (iv) incubated for 10 min with CCCP, incubated for 10 min with DTT, and then labeled with [35S]methionine for 20 min (lanes 4 and 8). Aliquots were immunoprecipitated with fumarase or aconitase antiserum and analyzed by SDS-PAGE followed by autoradiography. The arrows show the positions of precursor (p) and mature (m) fumarase.

The relationship between translation and translocation. Both plasmids harbor the GAL10 promoter; both the TEV protease and the Fum–TVS proteins are induced in galactose medium. It is important to point out that when expressed in yeast, these two fumarase derivatives are fully imported into mitochondria and do not distribute between the cytosol and mitochondria (not shown). This is expected, because, as we have previously reported, changes within the fumarase mature sequence or the addition of certain tags abolishes the fumarase dual subcellular distribution (18). This has allowed us to examine fumarase in the context of translocation without the complications of dual targeting of the protein. Cleavage of Fum92TVS by the TEV protease should cause the appearance of a shorter form of fumarase, as detected with specific fumarase antisera, whereas cleavage of Fum488TVS6H should result in disappearance of the His6 tag, as detected with specific His6 monoclonal antibodies (shown in Fig. 1B).

Previous studies in vivo and in vitro have shown that fumarase cannot be imported into mitochondria post-translationally (9, 10). To examine whether the same holds true for our fumarase derivatives (and as referred to in the Introduction), we pulse-labeled yeast expressing fumarase in the presence of the ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which dissipates the inner membrane potential, blocks translocation into mitochondria, and thus causes fumarase precursors to accumulate in the cytosol. Upon release of the translocation block using DTT, we were able to examine whether the cytosolic accumulated precursor can be translocated and processed in mitochondria. Both fumarase–TVS derivative precursors, which were accumulated in the presence of CCCP (Fig. 2, compare lanes 2 and 6 with lanes 1 and 5), could not be chased into mitochondria post-translationally following the treatment with DTT and reestablishment of membrane potential (Fig. 2, lanes 3 and 7). Thus, mature fumarase molecules were detected only when translation was coupled to import, either in the absence of CCCP or in the control experiment when cells were labeled after being treated with CCCP and incubated with DTT (Fig. 2, lanes 1 and 5 and lanes 4 and 8, respectively). As a control, we used aconitase, which can be imported post-translationally, and as seen in Fig. 2, mature aconitase molecules were detected after releasing the translation block (bottom, lane 3). The results indicate that these fumarase derivatives are not distributed as the wild type protein, yet they are fully targeted to mitochondria in a translation-translocation coupled manner, thereby providing an excellent model to investigate the latter.

Fumarase Is Not Cleaved during Import by Cytosolic TEV Protease—To examine whether the nascent chain of fumarase is exposed to the cytosol during import into mitochondria, we expressed each of the Fum–TVS derivatives in combination with a TEV protease localized either in the cytosol or in mitochondria. Western blot analysis was performed on cell extracts expressing these combinations employing fumarase antisera for detection of Fum92TVS and His6 monoclonal antibodies together with fumarase antisera for Fum488TVS6H. A single band corresponding to the expected 48-kDa fumarase was detected in yeast cells expressing Fum92TVS in the absence of a TEV protease (Fig. 3A, top, lane 1). When yeast cells co-expressed Fum92TVS and a mitochondrially targeted TEV protease, a second band appeared with a relative molecular mass corresponding to fumarase lacking its first 92 amino acids (Fig. 3A, top, lane 2). In contrast, co-expression of Fum92TVS with a TEV protease localized in the cytosol resulted in a single band without any cleavage products (Fig. 3A, top, lane 3). Matching results were obtained with Fum488TVS6H; co-expression of Fum488TVS6H with mitochondrial TEV protease resulted in the disappearance of the His6 tag (Fig. 3A, bottom, lane 2), whereas in the presence of the cytosolic TEV protease, the His6 tag remained intact (Fig. 3A, bottom, lane 3). The disappearance of the His6 tag is not the result of protein degradation, since all of the fumarase bands are similarly retained when probed with fumarase antisera (Fig. 3A, middle, lanes 1–3). Fum92TVS is only partially cleaved by the protease in contrast to Fum488TVS6H, which is fully cleaved. One explanation for this observation is that amino acid 92 of fumarase, located in the first domain of the protein based on its resolved crystal structure (19), is accessible for cleavage very transiently before becoming buried within the core of the rapidly folding protein. In any case, the proportion of cleaved to uncleaved Fum92TVS appears to be reproducibly constant under the conditions of our experiments.

One prediction is that Fum–TVS derivatives, which fail to be cleaved by the cytosolic TEV protease, due to import into mitochondria, should become substrates of the protease upon blocking of import. We blocked import into mitochondria using CCCP and found that both fumarase–TVS derivatives were cleaved by the cytosolic TEV protease. In the presence of a cytosolically disposed TEV, we detected the smaller form of the protein in the case of Fum92TVS (Fig. 3B, top, compare lanes 5
and 6) and observed the disappearance of the His<sub>6</sub> epitope in the case of Fum488TVS6H (Fig. 3B, bottom, compare lanes 5 and 11 to lanes 6 and 12). As expected, CCCP treatment prevented the accessibility of the fumarase-TVS derivatives to the mitochondrial TEV, and they accumulate as full-length (non-cleaved) precursors (Fig. 3B, top (compare lanes 3 and 4) and bottom (compare lanes 3 and 9 with lanes 4 and 10)). As a second control, we expressed fumarase-TVS derivatives lacking their MTS, so that they are localized in the cytosol. In this case, again, the fumarase-TVS derivatives were cleaved by the protease in the cytosol (Fig. 3C, top (lane 2 for Fum92TVS) and bottom (compare lanes 2 and 4 for Fum488TVS6H)).

In addition we checked aconitase, which, in contrast to fumarase, can undergo post-translational import into mitochondria (20). Aconitase harboring a TEV recognition site is cleaved by either the cytosolic or the mitochondrial TEV (not shown). Taken together, these results indicate that targeting to the mitochondria prevents the fumarase-TVS derivatives from being cleaved by the cytosolic TEV and rule out the possibility that the TEV protease is inactive in the cytosol or may not cleave these substrates in this compartment.

**Slowing Down Translocation Allows Cleavage of Fumarase by Cytosolic TEV**—The observation that the fumarase nascent chain is not exposed to the cytosol during import (as measured by cytosolic TEV cleavage) appears to contradict previous in vitro results, which show that at least the presequence of fumarase is exposed to the cytosol before import (14). One possible explanation for this could be that in vitro, import into mitochondria is not as efficient and rapid as in vivo. Therefore, we tested the effect of import rate in vivo on cleavage of the fumarase nascent chain in the cytosol during import.

Two TOM complex component mutants were used to slow down protein translocation into mitochondria, a tomo<sup>40</sup> temperature-sensitive (ts) mutant and null mim1<sup>ts</sup>. Tom40 forms the translocation channel across the outer membrane and is essential for cell viability under all growth conditions (21, 22). Our notion was to slow down mitochondrial protein import by limiting the function of the Tom complex with these mutations. The yeast tomo<sup>40</sup> ts mutant strain was grown at the permissive temperature (25 °C), and then the cells were incubated at the partially restrictive temperature of 30 °C for 8 h. The mim1<sup>ts</sup> deletion strain, which is fully viable, was grown at 30 °C. Total cell extracts were prepared from the cells of both strains, which were then analyzed by Western blotting as described above. The activity of Tom40 in the tomo<sup>40</sup> ts at 30 °C enabled full import and processing of both Fum92TVS and Fum488TVS6H (Fig. 4) (data not shown). Nevertheless, we detected cleavage of fumarase derivatives not only by the mitochondrial TEV but also the cytosol-targeted protease (Fig. 4, compare lanes 3 and 9 in the top and bottom). Similar results (not shown) were obtained in a mim1<sup>ts</sup> knock-out strain, which was shown to be impaired in the assembly of the TOM complex (23, 24). These results argue against the existence of a seal between the ribosome and the translocase during the import of fumarase. Alternatively, we suggest that fumarase is exposed to the cytosol during translation-coupled import and that slowing down
import provides the cytosolic TEV with ample time to cleave the nascent chain prior to translocation. To show that import is actually slowed down in our mutant strains, we developed a strategy for following the kinetics of fumarase import into mitochondria. This is based on the cleavage of incoming nascent chains by mitochondrial TEV. Yeast co-expressing fumarase-TVS derivatives and the mitochondrial targeted TEV protease were pulse-labeled with [35S]methionine/cysteine for 2 min and chased for 0, 1, 3, 5, and 10 min. Total cell extracts were prepared and analyzed as described in the legend to Fig. 3. B, the tom40 ts mutation slows down fumarase translocation into mitochondria. Induced cultures of the tom40 ts mutants or its corresponding wild type (WT), expressing Fum488TVS6H together with a mitochondrial targeted TEV protease, were pulse-labeled and analyzed as described in A. Shown below is a graphic representation of the accumulation of cleaved fumarase forms as a function of the chase period as deduced from the gels above.

FIGURE 5. A, cleavage of fumarase by TEV protease in mitochondria (Mit) occurs during or immediately after translation. Induced cultures of yeast expressing Fum92TVS or Fum488TVS6H with or without the mitochondrially targeted TEV protease were pulse-labeled with [35S]methionine/cysteine for 2 min, cold methionine/cysteine and cycloheximide were added to initiate the chase, and aliquots were taken at the indicated times. Total cell extracts were prepared and analyzed as described in the legend to Fig. 3. B, the tom40 ts mutation slows down fumarase translocation into mitochondria. Induced cultures of the tom40 ts mutants or its corresponding wild type (WT), expressing Fum488TVS6H together with a mitochondrial targeted TEV protease, were pulse-labeled and analyzed as described in A. Shown below is a graphic representation of the accumulation of cleaved fumarase forms as a function of the chase period as deduced from the gels above.

We also slowed down the translocation of fumarase into mitochondria by incubating yeast cells at lower temperatures. Cells co-expressing Fum92TVS or Fum488TVS6H with the TEV protease either in the cytosol or mitochondria were grown at 30 °C and then incubated for an additional 24 h at 22, 20, or 16 °C. Total cell extracts were prepared, and Western blot analysis was performed as described above. Fig. 6 shows that lowering the temperature enabled cytosolic cleavage of both Fum92TVS (top, lanes 7–9) and Fum488TVS6H (compare lanes 7–9 in the middle and bottom panels) by TEV. In both cases, lowering the incubation temperature was concomitant with a higher proportion of molecules cleaved by the cytosolic protease (Fig. 6, top and middle, compare lanes 7–9).

The membrane potential across the inner membrane is essential for protein translocation into the matrix. CCCP, a proton ionophore, can at the appropriate concentration dissipate this membrane potential and block import in vivo (25). Our notion was that a low concentration of CCCP, which does not fully block the import into mitochondria, should cause a reduction of the inner membrane potential and slow down protein translocation. Following calibration experiments, we determined that 0.25 μM CCCP does not arrest but slows down fumarase translocation. Yeast cells expressing Fum488TVS6H and the TEV protease in the cytosol were grown in glucose medium, washed, suspended in galactose medium, and then incubated for 8 h in the presence of 0.25 μM CCCP. Cell extracts were prepared and analyzed by Western blotting (Fig. 6B), showing that all the fumarase molecules were processed by mitochondrial processing peptidase to their mature forms in the presence of 0.25 μM CCCP. Nevertheless, under these conditions, almost all fumarase molecules are cleaved by cytosolic
Fumarase Cleavage by Cytosolic TEV Protease during Import

FIGURE 6. A, growth at low temperatures enables cleavage of fumarase by cytosolic TEV protease during import. Yeast expressing Fum92TVS or Fum488TVS6H were grown at 30 °C and then incubated for an additional 18 h at the indicated temperature. Total cell extracts were analyzed by Western blotting using the indicated antisera. The arrows show positions of the mature (m) and cleaved (c) forms of fumarase. B, partial dissipation of the membrane potential enables cleavage of the nascent chain by the cytosolic TEV protease. Wild type yeasts harboring Fum488TVS6H and either mitochondrial (Mit) or cytosolic (Cyt) TEV expression vectors were grown in glucose medium, washed, and resuspended in galactose medium and then incubated for 8 h in the presence or absence of 0.25 μM CCCP. Total cell extracts were analyzed by Western blotting using the indicated antisera.

TEV protease, indicating that fumarase is exposed to the cytosol during import (Fig. 6B, compare lane 6 in the lower and upper panels). These results strongly support our suggestion that the translocation rate determines the extent to which fumarase nascent chains are exposed to cytosolic cleavage during import.

Slowing Down both Translation and Translocation Annuls Fumarase Cleavage by Cytosolic TEV Protease during Import—Since slowing down translocation exposes fumarase to cytosolic TEV, it was important to test whether the rate of translation can affect this co-translational exposure. For this end, we used sublethal concentrations of the protein synthesis inhibitor cycloheximide (CHX). We assumed that these concentrations would allow a slow down rather than a complete block of translation in vivo. Cells co-expressing Fum92TVS or Fum488TVS6H with TEV protease either in the mitochondria or the cytosol were grown in galactose medium, and CHX was added to final concentrations of 0, 100, and 200 ng/ml. Total cell extracts were prepared and immunoprecipitated with fumarase antiserum or anti-His6 tag monoclonal antibodies as described above. As shown in Fig. 7A, 100 and 200 ng/ml CHX reduced translation by 17–24 and 29–48%, respectively (lanes 1–3 in both panels). Obviously, slowing down translation did not block cleavage of either Fum92TVS or Fum488TVS6H by mitochondrial TEV (lanes 4–6). Notably, slowing down translation did not enable cleavage by cytosolic TEV (lanes 7–9). These results suggest that the rate of translation does not affect the import rate and therefore under normal conditions does not affect the exposure of fumarase to the cytosol during import. If, however, translation is slowed down to allow cleavage of fumarase by cytosolic TEV prior to import (as shown above), we hypothesized that lowering the translation rate could eliminate this cleavage. Yeasts were grown in the presence of 0.25 μM CCCP, which slows import, and 100 ng/ml CHX, which slows translation. Total cell extracts were prepared and analyzed by Western blot as described above. As shown in Fig. 7B, slowing down the translation of Fum488TVS6H whose translation had been slowed down abolishes its cleavage by the cytosolic TEV protease (compare bands in lane 6 with those in lane 3). Slowing translation, thereby reversing the effect of slowing down translation, is expected only when synthesis and transport are functionally linked and therefore strongly supports a translation-coupled mode of fumarase import.

Finally, since our results suggest an interplay between translation and import into mitochondria, we questioned whether ribosome-associated factors may affect the accessibility of
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Fumarase nascent chains to cytosolic TEV cleavage. The nascent polypeptide-associated complex (NAC) had originally been identified in mammalian cells as a ribosome-associated factor that interacts with nascent polypeptides (26, 27). Further studies had suggested that the ribosome interacts with the mitochondrial surface and that the NAC plays a role in this interaction (28, 29). Therefore, we utilized a NAC null strain (egd1Δ, egd2Δ) (28) in which we co-expressed the fumarase TVS derivatives (Fum92TVS and Fum488TVS6H) together with the cytosolic or mitochondrial TEV protease. As shown in Fig. 8, a null NAC background enables cleavage of Fum92TVS (top, compare lanes 6 and 3) and Fum488TVS6H (bottom, compare lanes 6 and 3) by cytosolic TEV protease, whereas these substrates remained intact in the isogenic wild type strain (top lane 3 and middle lanes 3 and 6). The kinetics of the import, in the NAC null strain, were examined as described above and found to be slower (not shown). However, a full understanding of what happens in this mutant remains to be determined.

DISCUSSION

The term “co-translational translocation” immediately brings to mind a picture of the ribosome intimately sitting on a translocon, thereby feeding the translating polypeptide into a membrane channel. The coupling between translation and translocation in this ER-like scenario is not accidental, since in this case, translation is arrested until the ribosome is associated with the translocon. Furthermore, the signal recognition particle and signal recognition particle receptor complexes are dedicated to achieving the physical and functional coupling between the ribosome and translocon (30). Whether co-translational import into the ER involves a seal between the ribosome and the translocon is controversial (31–33), yet functional coupling between translation and translocation is widely accepted. In contrast to the ER, in which the mechanism of co-translational import has been thoroughly tackled, the same question of cotranslational import into mitochondria remains obscure. Here, we have focused on fumarase import as a model for translation-coupled import into mitochondria. Although fumarase cannot be imported post-translationally, in this study, we show that in vivo fumarase is exposed to TEV protease in the cytosol during import. This result is consistent with the requirement for cytosolic chaperones of the Ssa (Hsp70) family for fumarase import in vivo, suggesting that they interact with the protein in the cytosol prior to or during translocation (14, 18). This is also consistent with translation-coupled import in vitro, in which the presequence of fumarase is accessible to cleavage by externally added mitochondrial processing peptidase en route to mitochondria (14).

The novel feature that we propose in this study is a time frame for this “translation-coupled import” and a relationship between the translating ribosome and the translocon during in vivo import. Fumarase is not cleaved by cytosolic TEV protease during import (Fig. 9, Normal) unless translocation is slowed down by a mutation in a Tom complex component, by reducing the potential across the inner membrane, or by reducing the growth temperature (Fig. 9, Relaxed). From this, we can conclude that the time frame of import is in the order of TEV enzyme kinetics. We have found that slowing down or even stopping translocation into mitochondria does not affect the translation of fumarase (34). Here we show that under normal conditions, slowing translation does not affect exposure of fumarase to cytosolic TEV during import. Nevertheless, if translocation is slowed down, this causes exposure of fumarase to cytosolic TEV during import, which can be reversed by slowing down translation (Fig. 9, reversal of relaxed to normal). From this, we learn that the two processes, translation and translocation, are not directly dependent on one another, but the relative rate at which they occur determines the exposure of the translating-translocating polypeptide to TEV in the cytosol during import. In other words, if translation is faster
than translocation, the surplus of “not yet” translocated polypeptides can be cleaved by cytosolic TEV protease.

One possibility that we favor is that the import of fumarase occurs while the nascent chain is still associated with the ribosome based on (i) the strict requirement of translation coupled import of fumarase in vivo and in vitro, (ii) the extremely fast kinetics of import and processing of fumarase in vivo (9, 10), (iii) the results of this study, in which we show that fumarase cannot be cleaved by cytosolic TEV protease unless translocation is slowed down and that this may be reversed by reduced translocation, and (iv) the finding that NAC, a ribosomal associated complex, can affect exposure of fumarase to cytosolic TEV protease during import. This latter finding is consistent with the possibility that NAC mediates the interaction between the ribosome and translocon, which in turn affects translocation efficiency of substrates via translation-coupled import (28). In this regard, it is worth mentioning the known affinity of the ribosome for the TOM complex and the mitochondrial surface (6–8, 28, 29).

How is translation-coupled import of fumarase ensured? In other words how is the ribosome (translating fumarase) situated on, or at least in the vicinity of, the translocon in order to guarantee translocation into mitochondria rather than precursor folding into an irreversible import incompetent state in the cytosol? This latter fate in vivo and in vitro indeed occurs rapidly if fumarase is translated in the absence of mitochondrial import (9, 14). A number of mechanisms have been suggested to provide the feasibility of translation-coupled import: (i) targeting of mRNA to mitochondria (8, 35); (ii) efficient MTS-mediated targeting of nascent polypeptides to mitochondria (29); and (iii) the existence of unidentified ribosome targeting apparatus like the signal recognition particle/signal recognition particle receptor in the ER.

Finally, this study presents an experimental system for determining exposure of nascent chains during import and evaluating mitochondrial protein import kinetics in vivo. We have obtained analogous results for Fum92TEV and Fum488TEV6h, which are situated toward the N terminus and at the C terminus of fumarase, respectively. We have used this experimental approach to describe what appears to be a novel version of translation-coupled import of a protein into mitochondria.

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