Requirement of different mitochondrial targeting sequences of the yeast mitochondrial transcription factor Mtf1p when synthesized in alternative translation systems

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Mitochondrial (mt) translocation of the nuclearly encoded mt transcription factor Mtf1p appears to occur independent of a cleavable presequence, mt receptor, mt membrane potential or ATP [Biswas and Getz (2002) *J. Biol. Chem.* **277**, 45704–45714]. To understand further the import strategy of Mtf1p, we investigated the import of the wild-type and N-terminal-truncated Mtf1p mutants synthesized in two different *in vitro* translation systems. These Mtf1p derivatives were generated either in the RRL (rabbit reticulocyte lysate) or in the WGE (wheat germ extract) translation system. Under the *in vitro* import conditions, the RRL-synthesized full-length Mtf1p but not the N-terminal-truncated Mtf1p product was efficiently imported into mitochondria, suggesting that the N-terminal sequence is important for its import. On the other hand, when these Mtf1p products were generated in the WGE system,

surprisingly, the N-terminal-truncated products, but not the full-length protein, were effectively translocated into mitochondria. Despite these differences between the translation systems, in both cases, import occurs at a low temperature and has no requirement for a trypsin-sensitive mt receptor, mt membrane potential or ATP hydrolysis. Together, these observations suggest that, in the presence of certain cytoplasmic factors (derived from either RRL or WGE), Mtf1p is capable of using alternative import signals present in different regions of the protein. This appears to be the first example of usage of different targeting sequences for the transport of a single mt protein into the mt matrix.

Key words: mitochondria, protein import, *Saccharomyces cerevisiae*, transcription factor.

INTRODUCTION

The mitochondrial (mt) genome encodes a few mt proteins, but most of the mt proteins (approx. 95 %) including the mt transcription/translational machinery are nuclear gene products, which are synthesized in the cytosol and then imported into mitochondria [1–4]. Many of the nuclear DNA-encoded mt proteins are synthesized as precursors with N-terminal mt-targeting presequences, which are often removed after import [5,6]. Protein import through the translocation channels of the mt outer membrane [TOM (translocase of outer membrane)] and inner membrane [TIM (translocase of inner membrane)] requires energy from ATP hydrolysis, an electrochemical potential across the mt inner membrane and the participation of cytoplasmic and mt-specific chaperones [5–8]. Some proteins are imported co-translationally under *in vivo* [9] and *in vitro* conditions [6], whereas others are translocated post-translationally [6–9]. The mt preproteins may differ in the extent to which they are folded after presentation to the mitochondria. During import, most mt precursor proteins are partially unfolded [10,11]. However, a few precursor proteins may be in their native conformation after presentation to mitochondria, which then unfold these proteins without requiring ATP hydrolysis outside the mitochondria [12]. On the other hand, import of many other mt precursors requires extra-mt ATP, probably for their release from complexes with cytosolic chaperones [13,14]. It is probable that the latter group of mt preproteins is delivered from cytoplasmic ribosomes directly to the translocation machinery while still in a partially unfolded state. For example, the folding of precursor aspartate aminotransferase

from rat liver is inhibited by cytosolic factors when synthesized in a cell-free extract. This unfolded precursor is readily imported into the mitochondria, whereas the folded protein in the absence of cytosolic factors can no longer be imported [13].

A cleavable N-terminal presequence is present in most of the mt precursor proteins studied so far and this presequence is involved in many steps of mt protein import. Several mt proteins [15–20] including nuclearly encoded mt transcription factor Mtf1p [21–23] are imported into the mitochondria without such a presequence. These proteins use an internal or C-terminal signal sequence. Mtf1p also uses an extended sequence including the C-terminal region for its import, which appears to occur independent of general import factors [23]. We have demonstrated previously by sub-mt fractionation that, under these *in vitro* import conditions, Mtf1p is indeed translocated to the same mt sites as the endogenous protein [23]. In other words, a substantial proportion of Mtf1p is found in the matrix, where endogenous Mtf1p is also found. Mtf1p might well represent an example of a group of proteins not yet widely studied that is imported without a presequence. We have suggested previously that, after or during *in vitro* translation, Mtf1p assumes an import-competent conformation such that it bypasses the need for the oft-required membrane potential and mt outer-membrane receptor. The present study has been pursued for a better understanding of the non-conventional import mechanism of Mtf1p. We report here that the full-length and truncated Mtf1p may form alternative import-competent conformations involving more than one region of the protein. We tested the possibility that import-competent Mtf1p conformations depend on some cytoplasmic chaperone-like

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DTT, dithiothreitol; mt, mitochondrial; F₁β, β subunit of mt F₁-ATPase; NEM, N-ethylmaleimide; RRL, rabbit reticulocyte lysate; SRP, signal recognition particle; TNT, transcription–translation; TOM, translocase of outer membrane; WGE, wheat germ extract; WGEF, WGE factor.

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factor(s). We also show in the present study that the import of the mutant constructs, similar to the import of the wild-type Mtf1p, is independent of electrochemical gradient across the mt inner membrane, ATP hydrolysis, an mt receptor and/or a specific import temperature.

MATERIALS AND METHODS

Materials

The vector pGEM3, RRL (rabbit reticulocyte lysate) and WGE (wheat germ extract)-based TNT (transcription–translation)-coupled protein expression systems were purchased from Promega (Madison, WI, U.S.A.). [³⁵S]Methionine was obtained from Amersham Biosciences. The restriction enzymes and Vent polymerase were purchased from New England Biolabs. The oligonucleotide primers were prepared either at the University of Chicago core facilities or by Integrated DNA Technologies.

Isolation of mitochondria

Mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B by the method of Daum et al. [24]. The protein concentration was determined by the Bio-Rad Bradford method using BSA as a standard.

In vitro import of Mtf1p

The ³⁵S-labelled full-length and truncated Mtf1p products were generated *in vitro* using the RRL or WGE expression system as described earlier [23]. The quality and content of these Mtf1p products were examined by SDS/PAGE and fluorography before import assays.

An equal amount of radiolabelled Mtf1p was incubated with isolated yeast mitochondria in a standard 100 µl import reaction (0.6 M sorbitol, 10 mM MOPS, pH 7.2, 80 mM KCl, 2 mM ATP and 2 mM NADH) at 30 °C for 10 min unless otherwise indicated. After incubation, one-half of the import mix was treated with 20 µg of proteinase K on ice for 30 min to identify the imported Mtf1p while the other half was left untreated for the measurement of total mt-associated Mtf1p. After the addition of the protease inhibitor PMSF (1 mM final concentration), mitochondria were re-isolated by centrifugation, lysed in sample buffer (50 mM Tris/HCl, pH 6.0, 2 % SDS and 5 % 2-mercaptoethanol) and then subjected to SDS/PAGE (10–20 % gel). The radiolabelled band of Mtf1p was visualized by fluorography, and its intensity was then measured by densitometry.

RESULTS

Import of Mtf1p derivatives synthesized in the RRL or WGE translation system

Our earlier import studies relied on RRL-synthesized Mtf1p; however, we have compared two alternative Mtf1p expression systems (i.e. RRL and WGE translation kits; Promega) since yeast is not a typical animal or a plant. Furthermore, it has been reported that the cell lysate from different systems exhibits import-stimulating activity [25–29]. The potential influence of such stimulating factors may partly account for the unusual import of Mtf1p. We have compared the general import parameters of the full-length Mtf1p and an N-terminal-truncated Mtf1p. From the several deletion mutants, we have selected the Δ(2–147)Mtf1p carrying mainly the C-terminal half of the protein to illustrate the behaviour of these truncated mutants. With both RRL and WGE translation systems, the full-length *MTF1* template produced a single 43 kDa protein, whereas the Δ(2–147) con-

Figure 1 Differential *in vitro* import into mitochondria of full length and deleted Mtf1p generated in two translation systems

(A) *In vitro* import of the RRL- or WGE-synthesized full-length Mtf1p and the N-terminal-truncated Δ(2–147)Mtf1p. After incubation of Mtf1p with isolated yeast mitochondria at 30 °C for 10 min, one-half of the import mix was treated with proteinase K on ice for 30 min to remove the extra-mt Mtf1p while the other half was left untreated on ice to determine the total association of Mtf1p with mitochondria. The protease inhibitor PMSF (1 mM final concentration) was added, and mitochondria were re-isolated by centrifugation. The mt pellet was lysed in sample buffer and then subjected to SDS/PAGE (10–20 % gel). The radiolabelled Mtf1p on the gel was detected by fluorography. (B) Import assays were performed as above with some exceptions: lane 1, Mtf1p was incubated without mitochondria; lane 2, Mtf1p was incubated with mitochondria; lane 3, Mtf1p was incubated with mitochondria and then digested with proteinase K; lane 4, Mtf1p was incubated with mitochondria, followed by Triton X-100 treatment and proteinase K digestion.

struct generated two predominant polypeptides of 25 and 22 kDa molecular masses respectively (results not shown). According to the molecular mass predicted from the amino acid sequence, the 25 kDa peptide was the expected full-length Δ(2–147) product, whereas the 22 kDa polypeptide could be a smaller version of the same Δ(2–147) peptide generated from a second AUG codon 63 nt downstream of the first one.

To measure the import activity, an equal amount of each radioactive Mtf1p product (equal by radioactivity) was incubated with isolated yeast mitochondria. Interestingly, both full-length and Δ(2–147) associated with mitochondria at comparable levels [Figure 1A, lanes 1 and 5 of the wild-type and lanes 3 and 7 of the Δ(2–147)], but their import varied significantly depending on the translation system employed. For example, the full-length Mtf1p made in the RRL was readily imported into mitochondria (Figure 1A, lane 2), but this was not the case when the same protein was expressed in the WGE translation system (lane 6). On the other hand, the reverse situation applied to the Δ(2–147), which was well imported when translated in the WGE (Figure 1A, lane 8) but not in the RRL (lane 4).

To test the possibility that the protease resistance of Mtf1p observed in the import reaction may be due to self-aggregation of the translation product and/or its mt membrane association, Mtf1p was incubated with or without the mitochondria followed by centrifugation. It was found that Mtf1p alone in the import mix was not sedimented (Figure 1B, lane 1), whereas co-sedimentation of Mtf1p was observed after incubation with mitochondria (lane 2). These observations rule out the possibility of formation of insoluble self-aggregated Mtf1p during its incubation. Furthermore, in the absence of mitochondria, Mtf1p

Figure 2 Import activities of different N- and C-terminal-truncated Mtf1p mutants synthesized either in the RRL or WGE translation system

The highly charged regions of Mtf1p are indicated by the light shaded areas.

was fully digested by protease (results not shown). The results of Figure 1(A) also argue against an aggregation of Mtf1p that is independent of mitochondria, i.e. when synthesized in RRL, the full-length Mtf1p is resistant to proteolysis, whereas the N-terminal-truncated Mtf1p is fully digested. On the other hand, this latter product made in WGE is fully protected, in contrast with the full-length product that is readily digested. As described above, a large proportion of the mt-associated Mtf1p exhibited proteinase K resistance (Figure 1B, lane 3). However, the imported Mtf1p became sensitive to proteinase K when mitochondria were lysed with Triton X-100 after import reaction (Figure 1B, lane 4). It is worth noting here that we have demonstrated previously, by selective opening of the mt outer and inner membranes with osmotic swelling followed by probing with different antibodies directed against specific mt marker proteins, that Mtf1p is translocated *in vitro* to the same mt sites as the endogenous Mtf1p even under non-conventional import conditions [23].

In vitro import of different N- and C-terminal-deleted Mtf1p mutants

When synthesized in the WGE, the N-terminal deletion constructs Δ(2-147), Δ(2-194) and Δ(2-221) of Mtf1p but not other N-terminal [i.e. Δ(2-30), Δ(2-52) or Δ(2-100)] or C-terminal constructs [i.e. Δ(226-341), Δ(299-341) or Δ(325-341)] were readily imported into the mitochondria (Figure 2). On the other hand, when these proteins were synthesized in the RRL, the full-length and the C-terminal-truncated products [i.e. Δ(226-341), Δ(299-341) and Δ(325-341)] were well imported, whereas no import was observed with the N-terminal-deleted Mtf1p. Together, these observations suggest that the N-terminal sequence is critical for the import of Mtf1p made by RRL but is not sufficient for maximum import since deletion of the C-terminal sequence also reduced Mtf1p import efficiency. Interestingly, this N-terminal sequence is not required for import of some Mtf1p deletion products, e.g. the WGE-synthesized Δ(2-147) lacking the first 147 amino acid residues. Mtf1p, unlike many mt proteins studied so far, seems to have several stretches of its sequence capable of targeting it for import depending on the system employed for its biosynthesis. It appears that this protein might be capable of forming alternative competent conformations depending on some chaperone-like (or import factor) activity that differs between the two translation systems.

WGE stimulates import of Mtf1p products

The differences in the import of various Mtf1p sequences synthesized in the RRL and WGE systems respectively could be

attributable to factors that fix an import-competent conformation of these Mtf1p products. Most preproteins destined for the mitochondria need a partially unfolded conformation to cross the mt membrane [6,7,12,13,30]. This flexible import-competent structure of preprotein is maintained *in vivo* mainly by cytosolic chaperone-like import-stimulating factor(s) [6]. A similar role for chaperone-like factors in the *in vitro* translocation of some mt [11,13,27,29] and microsomal [25,26,28,31] proteins has also been reported. Thus we have considered the possibility of different import regulatory factor(s) in the cell-free RRL and WGE systems. For example, a cytosolic RRL factor not present in the WGE could interact with a specific N-terminal Mtf1p sequence, and perhaps enhance translocation of the full-length and C-terminal-truncated Mtf1p but not the N-terminal-truncated Mtf1p. On the other hand, WGE might have a different factor(s) [WGEF (WGE factor)], which could help Mtf1p to form an alternative import-competent conformation in the absence of its N-terminal sequence. To account for our observations, the productive interaction between WGEF and Mtf1p is anticipated only in the absence of its N-terminal sequence.

To address this issue further, we have performed mt import of Mtf1p in the presence or absence of WGE (or RRL) as a potential source of import regulatory factor(s). In these experiments, the full-length Mtf1p and Δ(2-147)Mtf1p were synthesized in the RRL or WGE system for 1 h and, then, protein translation was terminated by the addition of cycloheximide (final concentration, 20 μg/ml). To detect the import-stimulating activity in the RRL, the WGE-synthesized protein was incubated with mitochondria with or without the addition of 10 μl (approx. 1 mg) of RRL from the TNT kits per 100 μl import reaction (Figure 3). A reciprocal import experiment was also performed with the RRL-synthesized Mtf1p using WGE as the source of the putative import factor. These import experiments were set up to reproduce as closely as possible the conditions used for the import of Mtf1p synthesized *in vitro* in either RRL or WGE, i.e. the reactions containing mitochondria and the WGE-synthesized Δ(2-147)Mtf1p substrate were supplemented with a concentration of RRL at least comparable with that present in the import reaction set up with the protein translated with RRL and vice versa. The post-synthetic addition of RRL to the WGE-synthesized protein did not influence import of either full-length (Figure 3, lanes 1-3) or truncated Mtf1p products (lanes 7-9). The WGE-synthesized full-length Mtf1p remained import-incompetent even in the presence of RRL added during import. This is best explained by assuming that the putative rabbit reticulocyte factor must act to generate an import-competent conformation during translation, i.e. on the nascent translation product. To test this possibility, we attempted to synthesize the full-length Mtf1p with the WGE translation system in the presence of RRL supernatant, from which ribosomes have been removed by centrifugation. However, Mtf1p was not expressed in this WGE/RRL mixed translation system. In the other case, the presence of WGE in the import reaction of the RRL-synthesized full-length Mtf1p exhibited slight import inhibition (Figure 3, lanes 4-6). On the other hand, the import of Δ(2-147)Mtf1p (i.e. 25 and 22 kDa) synthesized in the RRL was enhanced severalfold in the presence of WGE (Figure 3, lanes 10-12). The RNase (RNase A1) added in this experiment appears to be required for the function of the putative WGEF (see below). This import stimulation was specific to the WGE since the post-translational addition of a comparable level of RRL protein did not alter the import of the Δ(2-147)Mtf1p. Interestingly, the import stimulation by WGE on Δ(2-147)Mtf1p was more pronounced in the presence of an RNase (Figure 3, lane 12), which was initially used to explore whether an RNA component of the WGEF might account for its stimulatory capacity as has been found

Figure 3 Import of the full-length Mtf1p and $\Delta(2-147)$ Mtf1p in the presence of WGE or RRL

Mtf1p was synthesized in the WGE (or RRL) system for 1 h and, then, protein synthesis was stopped by the addition of cycloheximide (final concentration, 20 μ g/ml). These WGE-synthesized (lanes 1–3 and 7–9) or RRL-synthesized proteins (lanes 4–6 and 10–12) were incubated with isolated mitochondria as described above in the presence or absence of ribosome-depleted RRL or WGE respectively as a source of potential import-stimulatory factor(s). RNase A1, which is required for the import-stimulatory activity of some WGE preparations (see Figure 3), was included in this import assay. After incubation, one-half of the import mix was treated with proteinase K (PK) to identify the imported Mtf1p (+PK, lower panel), whereas the other half was left untreated to determine the total mt association of Mtf1p (–PK, upper panel).

Figure 4 The RNase-dependent import-stimulatory activity in the WGE

Mt import of the RRL-synthesized $\Delta(2-147)$ Mtf1p was performed with isolated mitochondria either alone (lane 1) or containing WGE (lane 2), RNase A1 (lane 3), WGE + RNase A1 (lane 4–6), WGE + RNase A1 + RNasin (lanes 7–9) or WGE + RNasin (lane 10). PK, proteinase K.

with the SRP (signal recognition particle) of the endoplasmic reticulum protein translocation machinery [32]. With the recent batches of TNT kit (Promega), the import stimulation by WGE is observed only in the presence of an RNase. When mitochondria were preincubated with WGE, centrifuged to remove WGE and then incubated with RRL-synthesized $\Delta(2-147)$ Mtf1p, the import efficiency was not improved (results not shown). This suggests that WGEF might act on the $\Delta(2-147)$ Mtf1p conformation that makes the protein capable of import.

Import stimulation by WGE requires an RNase activity

The import stimulation by RNase (Figure 3) could be due to its enzymic activity or it could carry an impurity that is responsible for this stimulatory activity. We have used several RNase preparations from different sources, all of which yielded similar results. To demonstrate further the role of RNase activity in this import activation, the import assay was performed with WGE + RNase with or without RNasin (an RNase inhibitor). In the control experiments, neither WGE nor RNase alone exhibited import stimulation of the RRL-synthesized $\Delta(2-147)$ Mtf1p (Figure 4, lanes 2 and 3), whereas substantially enhanced import was noted in the presence of WGE and 1–10 μ g of RNase (lanes 4 and 5) but not in the presence of ≤ 0.1 μ g of RNase (lane 6). The stimulatory activity of RNase (1 μ g) in the presence of WGE was inhibited by RNasin (20 units; Figure 4, lane 8). This suggests that RNase activity is necessary for import-stimulatory function of the WGE.

Figure 5 The WGE carries a heat-sensitive soluble import factor

Import of the RRL-synthesized $\Delta(2-147)$ Mtf1p was performed in the presence of normal or pretreated WGE. Lanes 1–4, control assays with or without WGE; lane 5, import in the presence of WGE that was preheated at 95 °C for 10 min; lanes 6 and 7, import in the presence of supernatant and pellet respectively from the centrifugation of WGE at 130 000 g for 30 min. The WGE pellet was resuspended in an equal volume of import buffer. Lanes 8 and 9 are identical with lanes 6 and 7, except that RNase A1 was added to the assays of lanes 8 and 9. PK, proteinase K.

The WGEF is a soluble heat-sensitive import factor

We have further characterized the nature of the putative WGEF. The control experiments (Figure 5, lanes 1–4) duplicated those just described above (Figure 4). The RRL-synthesized $\Delta(2-147)$ Mtf1p exhibited no import activity (Figure 5, lower panel, lanes 1–3). However, in the presence of WGE/RNase A1 both peptides were imported into the mitochondria efficiently (Figure 5, lane 4), as expected. The preheating of WGE at 95 °C for 10 min decreased its import-stimulatory activity significantly (Figure 5, lane 5), indicating that the WGEF is heat-labile. When WGE was separated into soluble and insoluble components by centrifugation, the supernatant (Figure 5, lane 8) but not the pellet fraction (lane 9) retained most of the import-stimulatory activity. This suggests that the putative WGEF is a soluble heat-labile factor.

Do the full-length Mtf1p and $\Delta(2-147)$ Mtf1p follow similar import pathways?

The results described above suggested that the import of the full-length Mtf1p and that of the N-terminal-truncated $\Delta(2-147)$ Mtf1p may differ in some respects. To explore the extent to which these two proteins follow similar import pathways, we have studied the mt import of the WGE-synthesized $\Delta(2-147)$ Mtf1p

Figure 6 Mtf1p import into mitochondria with different concentrations of isolated yeast mitochondria

(A) Import of RRL-synthesized full-length Mtf1p and WGE-synthesized $\Delta(2-147)$ Mtf1p with different concentrations of isolated yeast mitochondria. Import was performed with different amounts of mitochondria (i.e. 0, 10, 25, 50, 100 or 200 μ g per 100 μ l reaction) under standard assay conditions. (B) Time course for Mtf1p import. Import was performed as above with 1 mg/ml mitochondria for different time periods. The imported protein was identified by SDS/PAGE and fluorography and then measured by densitometric scanning. Percentage import of each protein was calculated considering its maximum import (approx. 10 min) as 100%. ●, full-length Mtf1p; ○, $\Delta(2-147)$ Mtf1p. PK, proteinase K.

and that of the RRL-synthesized full-length Mtf1p in parallel. In the control experiments, $F_1\beta$ (β subunit of mt F_1 -ATPase) was used as an mt matrix protein that follows a conventional import pathway.

Import with different concentrations of mitochondria

There is evidence that the transfer of most mt matrix proteins from the cytosol into the mitochondria occurs at contact sites between the inner and outer mt membranes [9]. There are a limited number of translocation sites and a defined number of assemblies of the translocation machinery for proteins crossing the mt membrane [33]. Since the mt import of preproteins depends on the productive association of preproteins with the mt membrane, the import efficiency of mt proteins might vary depending on their initial interaction with the membrane receptors. These membrane sites might be saturated with different concentrations of import substrates depending on their affinity. To examine this interaction between Mtf1p products and mitochondria, import was performed with different concentrations of isolated mitochondria. The 100 μ l reaction contained 5 μ l of labelled protein but variable amounts of isolated mitochondria (i.e. 0, 10, 25, 50, 100 or 200 μ g per 100 μ l reaction) incubated for 10 min at 30 °C. Under these assay conditions, the maximal level of mt association/import of the full-length protein was observed at approx. 0.5 mg/ml (or 50 μ g/100 μ l) mt concentration (Figure 6A, upper panel, lanes 3 and 4). On the other hand, these mt concentrations had a smaller effect on the import of $\Delta(2-147)$ (Figure 6A, lower panel).

Time course for *in vitro* import

To determine the time period required for maximum import of the full-length Mtf1p and the $\Delta(2-147)$ Mtf1p, a time course (0, 3, 5, 10, 15 and 30 min) experiment was performed with 1.0 mg/ml mt concentration. Import of both Mtf1p products between 0 and 5 min appears to be linear with time, reaching a plateau within 10 min (Figure 6B). The import rates of both Mtf1p products are comparable. The rest of the Mtf1p import reactions was performed with 1.0 mg/ml mitochondria for 10 min at 30 °C unless otherwise stated.

Mtf1p import without ATP

The widely studied pathway for protein import into mitochondria requires ATP to not only maintain a chaperone-mediated import-competent conformation of precursor in the cytosol but also translocate preprotein through the inner membrane into the mt matrix [6–8]. Import of the full-length Mtf1p, the N-terminal-truncated Mtf1p and the control protein $F_1\beta$ was examined in the absence of ATP. To remove the endogenous ATP, the translation mixture containing the labelled protein and the isolated mitochondria were separately treated with apyrase before import. Import was started by mixing the apyrase-treated mitochondria and the *in vitro* translation products. In the absence of ATP, the mt association was not affected with any of these proteins; however, import of $F_1\beta$ was drastically reduced without ATP, as anticipated (Figure 7A, lane 2). On the other hand, both full-length (Figure 7B, lane 2) and the truncated Mtf1p product (Figure 7C, lane 2) were effectively imported into mitochondria. This suggests that there is little or no ATP requirement for import of either of these two Mtf1p products.

Mtf1p import without mt membrane potential

The mt membrane potential facilitates translocation of most mt matrix proteins across the inner membrane [6,7]. To test the effect of membrane potential on the import capacity of Mtf1p, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; an uncoupler of oxidative phosphorylation) or valinomycin (K^+ ionophore) was used to discharge the mt membrane potential. As a control, the import of $F_1\beta$ was also studied. In the presence of CCCP or valinomycin, $F_1\beta$ was associated with mitochondria but not translocated into protease-resistant mt compartment (Figure 7A, lanes 3 and 4). However, both the wild-type Mtf1p and deletion mutant of Mtf1p were imported into the mitochondria (Figures 7B and 7C, lanes 3 and 4). This suggests that the mt import of Mtf1p or its N-terminal-truncated mutant requires no mt membrane potential.

Mtf1p import at low temperature

Temperature is a critical factor for *in vitro* mt protein import [11,34]. Temperatures lower than 20 °C slow down the import process of many mt proteins and also trap some translocation intermediates in transit through the mt membrane [11,34]. To explore the effect of reaction temperature on Mtf1p import, the *in vitro* assay was also performed on ice (3 °C). The mt association of all three peptides at this low temperature appeared to be similar to that at 30 °C (Figure 7, lane 5). However, the import of $F_1\beta$ (Figure 7A, lane 5) but not that of the Mtf1p products (Figures 7B and 7C, lane 5) was drastically reduced on ice.

Mtf1p import without trypsin-sensitive mt outer-membrane receptor

In an early step of mt protein import, the receptor proteins on the outer surface of mitochondria [MOM70 (mt outer-membrane

Figure 7 Mtf1p import into mitochondria in the absence of general import factors

Import of RRL-synthesized full-length Mtf1p and WGE-synthesized $\Delta(2-147)$ Mtf1p was examined in the absence of general import factors. Lane 1, import under standard assay conditions; lane 2, import in the absence of ATP (i.e. before import assay, the isolated mitochondria and the Mtf1p translation products were separately treated with 5 units of apyrase at 25 °C for 20 min to remove endogenous ATP). Lanes 3 and 4, import in the absence of mt membrane potential (i.e. mt membrane potential was destroyed by pretreatment of mitochondria with 40 μ M CCCP or 5 μ M valinomycin); lane 5, import on ice (3 °C) (i.e. Mtf1p alone and import mix without Mtf1p were kept separately on ice-water for 15 min and then mixed together to start the import); lane 6, import with trypsin-pretreated mitochondria (mitochondria without outer-membrane receptors); lane 7, import in the presence of adriamycin. Mt import was performed as above, and the import measurements were performed considering the total mt association of the full-length Mtf1p as 100%. PK, proteinase K.

receptor 70 kDa) and MOM19] recognize the mt precursor proteins to be imported [6,8]. These receptor proteins are extremely sensitive to mild protease (i.e. trypsin) treatment, whereas components of the general TOM and TIM channel complexes are resistant to it [35,36]. Pretreatment of mitochondria with trypsin generally inhibits import of mt preproteins. To examine Mtf1p import in the absence of these receptor proteins, *in vitro* import of Mtf1p and $F_1\beta$ was performed with trypsin-treated mitochondria. Both Mtf1p products were imported into these protease-treated mitochondria, albeit to a lower extent compared with untreated mitochondria (Figures 7B and 7C, lane 6), whereas $F_1\beta$ was not imported at all (Figure 7A, lane 6) since $F_1\beta$ requires such a receptor. This suggests that Mtf1p does not have a critical requirement for a protease-sensitive mt outer-membrane 'receptor'.

Mtf1p import in the presence of the phospholipid-binding compound adriamycin

Acidic phospholipids in the mt outer membrane may facilitate both unfolding and import of mt precursor protein [37]. Cardio-

lipin, which is an acidic phospholipid enriched in mt membranes [38,39], is found to be required for efficient binding and unfolding of precursors with the mitochondria as well as with phospholipid vesicles [39,40]. It has also been reported that mt protein translocation is blocked in the presence of an acidic phospholipid-binding drug, adriamycin [40]. To test whether mt phospholipids may represent the mt surface components responsible for recognition of Mtf1p and its deletion mutant, mitochondria were pretreated with adriamycin. Pretreatment of mitochondria with adriamycin inhibited import of $F_1\beta$ (Figure 7A, lane 7). This is consistent with a previous report that $F_1\beta$ efficiently binds to liposomes containing cardiolipin [39]. In contrast, import of Mtf1p and its deletion mutant into the adriamycin-treated mitochondria was unchanged (Figures 7B and 7C, lane 7), suggesting that Mtf1p import is independent of adriamycin-susceptible mt membrane phospholipids. This corroborates our finding that pretreatment of mitochondria with different phospholipases (i.e. bovine pancreatic phospholipase A_2 , Clostridium phospholipase C, Streptomyces phospholipase D, human placenta sphingomyelinase at 37 °C for 2 h) did not affect the mt uptake of Mtf1p (results not shown).

Mtf1p import in the presence of a reducing agent or cation

Protein import into isolated mitochondria or chloroplasts is inhibited in the presence of the SH group-binding agent DTT (dithiothreitol; a reducing agent) [41,42], NEM (*N*-ethylmaleimide; a membrane-permeant alkylating agent) [42,43] or Cu^{2+} (an oxidizing cross-linker) [42,44]. Since the import behaviour of the full-length and $\Delta(2-147)$ in the above-described experiments was indistinguishable, Mtf1p import was also performed in the presence of these compounds to test the possibility that they exhibit differential sensitivity to these agents. The addition of NEM (2 mM) or DTT (4 mM) did not affect import of full-length Mtf1p or $\Delta(2-147)$ Mtf1p (Figures 8B, lanes 2 and 3, and 8C, lanes 2 and 3 respectively). This suggests that SH groups are apparently not involved in the import of Mtf1p. To determine whether Mtf1p requires any cation for its entry into the mt matrix, import was performed with or without the metal-chelating agents EDTA (i.e. chelator of Mg^{2+} , Mn^{2+} , Zn^{2+} or Ca^{2+}) and 1,10-phenanthroline (i.e. chelator of Fe^{2+} and Zn^{2+}). Import of neither full-length Mtf1p nor $\Delta(2-147)$ Mtf1p was inhibited by these metal chelators (Figures 8B and 8C, lane 4), whereas import of $F_1\beta$ was substantially affected (Figure 8A). In the presence of Cu^{2+} ions, there was no difference in the mt association and import of Mtf1p or its truncated mutant (Figures 8B and 8C, lane 5).

DISCUSSION

In the present study, we extend the results of our previous studies on mt translocation of the yeast mt transcription factor Mtf1p. We have recently described the import of Mtf1p into isolated mitochondria, which appears to be unique among previously described mt import mechanisms [23]. It occurs independent of a cleavable N-terminal presequence, a proteinaceous mt receptor or the energy state of the mitochondria (not inhibited by agents that discharge the electrochemical gradient). This sequence was also capable of carrying a non-mt protein, dihydrofolate reductase, into the mitochondria by a similar mechanism [23]. We postulated that during translation and in the presence of the chaperones endogenous to RRL, this protein assumes an import-competent conformation that could be readily disrupted by urea denaturation [23]. Although the earlier deletion analysis did not define the targeting sequence of Mtf1p, the 30-amino-acid N-terminal region

Figure 8 Mtf1p import into mitochondria in the presence of SH reagents or metal chelators

Import of RRL-synthesized full-length Mtf1p and WGE-synthesized $\Delta(2-147)$ Mtf1p in the presence of SH group-binding agents NEM (lane 2) or DTT (lane 3), metal chelators EDTA and 1,10-phenanthroline (lane 4) or bivalent Cu^{2+} cations (lane 5). PK, proteinase K.

of Mtf1p appeared to be required for mt targeting, but this sequence was not sufficient since deletions elsewhere in the molecule also impaired targeting [23]. In the present study, we show that when this protein was translated in the WGE, the sequence requirements for import appeared to be quite different. We report here the import of mutant deletion constructs [i.e. $\Delta(2-147)$ -Mtf1p] that could not be imported when translated in RRL. When almost half the protein sequence was deleted from the N-terminus, the residual protein synthesized in the WGE was quite effectively imported into the mitochondria. This import of $\Delta(2-147)$, although not quite as efficient as that of the intact protein, again occurred independent of general import requirements (receptor, energy state of mitochondria etc.). Analysis of a variety of Mtf1p deletion mutants indicates that the sequence requirements for its import from RRL and WGE translation systems are quite different. The N-terminal sequence appears to be relatively insignificant for $\Delta(2-147)$ import when synthesized in the WGE translation system. This observation suggests that the import-competent conformation that allows Mtf1p import when translated in the RRL is probably not required or generated in the WGE translation system. The absence of the first 147 amino acids in the $\Delta(2-147)$ may result in a conformation exposing a stretch of positively charged residues at its N-terminus, whereas the full-length protein carrying the N-terminal sequence might have a different structural conformation. The simplest hypothesis to account for these unusual results is that RRL and WGE contain different sets of chaperones that bind to the Mtf1p, resulting in two different import-competent conformations. The WGE may promote an import-competent conformation of the truncated mutant, while inhibiting the formation of an import-competent

conformation of the full-length Mtf1p. It is not clear whether these properties of the WGE are contributed by a single or multiple protein(s).

These findings raise a number of issues. What are the target sequences within Mtf1p that allow for import from an RRL or WGE translation system. Together with our earlier observations [23], the present study suggests that, when translated in RRL, the N-terminus is required but is not sufficient for efficient import. It also appears that the C-terminal 122-amino-acid region, when synthesized in the WGE, is sufficient for good import. Does this C-terminal portion also assume an import-competent conformation that depends on a stimulating chaperone present in WGE? The findings described in Figures 3 and 4 do indicate that a factor(s) present in WGE, when added post-translationally to the C-terminal half of the protein translated in RRL, does facilitate the import of the translated protein. The role played by the enzymic activity of an RNase in promoting the import capacity of the WGE-synthesized (2-147)Mtf1p suggests that the WGEF might be a ribonucleoprotein similar to the SRP used for endoplasmic reticulum protein translocation [32]. However, unlike the RNA subunit of SRP, which functions as an activator, the putative RNA component of WGE serves as a constraint on its activity. The use of RNase alone in the import assay has no influence (Figure 3, lane 5). The argument that this WGEF(s) is chaperone-like stems from the fact that it acts post-translationally and is found in the supernatant of the centrifuged WGE (Figure 5). It remains to be established whether any of these import-competent conformations of Mtf1p use the general import pathway and/or co-opt any of the components of the general import complexes. This can be assessed in appropriate import-defective yeast mutants [45].

The fact that the first 147 amino acids must be removed before we can observe a significant import from a wheat germ translation mix argues for the presence of an inhibitory sequence or conformation that is incompatible with import from this extract. An examination of the Mtf1p sequence suggests a possible basis for this inhibitory conformation. Between residues 157 and 197, there are ten basic residues without any acidic residue. In the segment encompassing residues 100-147, there are ten acidic residues with only two basic ones. It is possible that these two domains interact by salt bridges to construct a conformation that is unable to be imported in the presence of wheat germ chaperones. These results also suggest that the profile of chaperone-like molecules differs significantly between the RRL and WGE. There have been other reports suggesting that some factors present in the translation systems may influence import differently. For example, an efficient mt uptake of *Drosophila* apocytochrome *c* was noticed only in the presence of a WGEF(s) [27]. The nuclear-coded yeast mt Δ pyrroline-5-carboxylate dehydrogenase (Put 2) is translocated into mitochondria when translated in the RRL system but not in the WGE system [28]. Cytosolic factors present in the rat liver appear to inhibit the translocation of precursor aspartate aminotransferase [13]. The chaperone Hsc70 and the DNAJ homologues (i.e. dj2 or dj3) are required for the import of *in vitro*-synthesized preornithine decarboxylase into mammalian mitochondria [46]. However, if this protein is completely synthesized in the absence of these chaperones, it cannot be imported even after their post-translational addition [46].

The import system of Mtf1p and its truncated products, independent of general import requirements, is unique in the sense that no other protein has so far been described that is quite so independent of all the features of this system. There are some mt proteins that are independent of one or more features of the general import system but none of them are completely independent. Apocytochrome *c* [19], AAC (ADP/ATP carrier protein) [15] and BCS1 [17] all seem to lack the N-terminal targeting sequence.

The mt targeting signal of these proteins is probably located in the internal region or in the C-terminal half of the molecule. The mt targeting sequence of CCHL also appears to be in the third-quarter of the protein [16]. Others have an N-terminal targeting sequence that is not cleaved, e.g. the 70 kDa outer-membrane protein [47], 3-oxoacyl-CoA thiolase [48], GTP-AMP phosphotransferase [49] and adenylate kinase (Aky2p) in yeast [50]. On the other hand, a class of mt [51,52] or chloroplast [53] proteins carries a cleavable presequence that may enhance import but is not essential for organelle targeting. Apocytochrome *c* is translocated into the mitochondria in the absence of a cleavable mt targeting sequence and without a protease-sensitive receptor, ATP or the mt membrane potential [19,54]. Mt translocation of subunit Va of cytochrome *c* oxidase into the mt inner membrane appears to be independent of a surface receptor and requires a very low level of ATP [55]. Bcl2, whose import and membrane-insertion signals are located in a hydrophobic C-terminal segment, is imported into mitochondria in the absence of mt membrane potential and without protease-sensitive mt membrane components [18]. AAC translocation into the mt inner membrane also occurs independent of a cleavable presequence, ATP hydrolysis and mt-Hsp60, but requires an mt membrane potential [15]. Many of the proteins discussed here are outer/inner-membrane proteins as well as intermembrane space proteins, but very few proteins find their way to the matrix without general import requirements. It is worth noting that a receptor and/or temperature-independent cellular translocation of proteins has also been noticed with non-mt proteins. The human HIV TAT protein or its recombinant chimera carrying an 11-residue (YGRKKRRQRRR) highly positively charged transduction domain of TAT protein moves into the cell in an energy-independent manner and without any receptor or transporter involvement [56]. The *Drosophila* Antennapedia homeoprotein is also internalized into cells in the absence of a specific receptor and even at a low temperature (i.e. 10 °C) [57,58]. Interestingly, all the three, yeast mt Mtf1p, HIV TAT and *Drosophila* homeoprotein, are transcription factors involved in gene-specific transcription of these species.

Does Mtf1p follow a novel import pathway? There are at least three different import routes operated for the nuclear-encoded mt matrix proteins [59]. At this point, we cannot say whether any of these pathways are used in whole or in part by Mtf1p for its transport into mitochondria. A pretreatment of mitochondria with trypsin, which removes the cytosolic domains of outer-membrane import receptors such as Tom20, Tom22 and Tom70, did not affect Mtf1p import, suggesting that Mtf1p translocation occurs independent of these surface receptor functions. It is also possible that the need for surface receptors in early steps of precursor-receptor interactions could be bypassed if Mtf1p uses its own membrane insertion activity as has been described for apocytochrome *c* [19,54] or HIV TAT protein [56]. The force for this membrane insertion might come from the trapped energy in the specific structure/conformation of Mtf1p. Similar to apocytochrome *c*, Mtf1p might diffuse through the mt outer membrane in a concentration-dependent manner. At this point, Mtf1p might be capable of moving bidirectionally across the mt membrane. However, after interacting with the mt membrane channel proteins (i.e. Tom7, Tom40, Tim23 and Tim40), Mtf1p might make a full commitment for its translocation into the mt matrix. Finally, Mtf1p may be sequestered within the mitochondria by interaction with an intra-mt component such as mt core polymerase and/or mt DNA. This could be analogous to apocytochrome *c*, which is sequestered in the intermembrane space of mitochondria after interaction with haem to form the haemoprotein.

Results presented in this study highlight the hitherto unrecognized variety of mechanisms that could support mt protein

import. We have also shown how these import mechanisms, in turn, could be influenced by a variety of cytosolic chaperones which are capable of playing different roles in facilitating or inhibiting the generation of conformations of mitochondrially destined proteins that are competent for import. It remains to be seen whether the unusual features of Mtf1p are unique and peculiar to this protein or whether they represent an example of a pathway followed by several mt proteins that have not yet been intensively studied. If this is the case, it might uncover a rich diversity of mechanisms that could be used for the specific and efficient targeting of a large number of nuclearly encoded mt proteins under different physiological conditions. Elucidation of these new translocation mechanisms could provide powerful strategies for the *in vivo* delivery of therapeutically important agents into the mitochondria.

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