A cryptic matrix targeting signal of the yeast ADP/ATP carrier normally inserted by the TIM22 complex is recognized by the TIM23 machinery

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The yeast ADP/ATP carrier (AAC) is a mitochondrial protein that is targeted to the inner membrane via the TIM10 and TIM22 translocase complexes. AAC is devoid of a typical mitochondrial targeting signal and its targeting and insertion are thought to be guided by internal amino acid sequences. Here we show that AAC contains a cryptic matrix targeting signal that can target up to two thirds of the N-terminal part of the protein to the matrix. This event is coordinated by the TIM23 translocase and displays all the features of the matrix-targeting pathway. However, in the context of the whole protein, this signal is ‘masked’ and rendered non-

functional as the polypeptide is targeted to the inner membrane via the TIM10 and TIM22 translocases. Our data suggest that after crossing the outer membrane the whole polypeptide chain of AAC is necessary to commit the precursor to the TIM22-mediated inner membrane insertion pathway.

Key words: ADP/ATP carrier, dihydrofolate reductase (DHFR) fusion, mitochondria biogenesis, protein translocation, translocase of the inner membrane.

INTRODUCTION

In eukaryotic cells 98.9% of mitochondrial proteins are synthesized in the cytosol, and are then translocated to one of the four mitochondrial compartments depending on their targeting signal (reviewed in [1]). To cope with this large number of different polypeptides, mitochondria have a variety of translocation systems and pathways. The majority of precursor proteins are targeted via two distinct pathways: the TIM23 pathway used mainly by matrix-targeted proteins containing an N-terminal, cleavable presequence and the TIM22 pathway used by inner membrane (IM) proteins with internal targeting signals. The latter accounts for the translocation of the most abundant proteins of the IM, the family of the metabolite carriers like the ADP/ATP carrier (AAC). This family contains at least 34 different polypeptides in the yeast *Saccharomyces cerevisiae* [2]. Throughout the text we adopt the convention of using TIM and TOM (uppercase) to refer to protein complexes and Tim and Tom for individual proteins.

Among all metabolite carriers, the import pathway of AAC has been studied in most detail and is divided into five stages [3]. In stage I, the precursor is translated in the cytosol and interacts with cytosolic chaperones that maintain it in a translocation-competent conformation [4]. Stage II, the precursor-cytosolic chaperones complex binds to a receptor complex (mainly Tom70) on the *cis* side of the outer membrane (OM) [5,6]. The recruitment of AAC by the Tom70 receptor involves a cooperative mechanism whereby each repeat module of AAC (approx. 100 residues) binds to one dimer of Tom70 receptor [7]. Transfer from this stage to the next one requires energy in the form of ATP hydrolysis [8]. In stage III, the AAC crosses the OM through the TOM channel in a loop conformation and is bound by the soluble, intermembrane space (IMS) 70 kDa complex made of Tim9 and Tim10 [5,9–12]. In stage IV, the precursor is targeted to the IM where it is inserted by the TIM22 complex [13,14]. This stage requires a membrane

potential ($\Delta\Psi$) across the IM [13]. Finally, in stage V, AAC dimerizes to a functional molecule [15].

On the other hand, proteins with a presequence also bind to molecular chaperones in the cytosol. At the *cis* side of the OM, they are then recognized by a different receptor complex containing mainly Tom20, and are then transferred to Tom22 [16,17]. The same TOM channel that also recognizes carrier proteins allows passage of presequence containing precursors through the OM. In the *trans* side of the OM, translocation is mediated by the IMS segment of Tom22 [18], Tim23 [19] and Tim50 [20–22]. Passage across the IM then occurs through the distinct TIM23 channel and is facilitated by the import motor containing Tim44, Tim14 and Hsp70 that trap precursors and target them to the matrix [23–25]. Once in the matrix, the presequence is cleaved by the mitochondrial processing peptidase [26].

For some precursors, import is ensured by a crossover mechanism between the above distinct pathways. Examples include the Tim22 and Tim54 proteins [27].

We wanted to determine if the internal targeting signals of AAC are also required to work in a cooperative manner (as for the recruitment of Tom70) to cross the IMS. For that aim we used a deletion strategy by fusing different fragments of AAC to a non-mitochondrial protein, the mouse dihydrofolate reductase (DHFR). Either one, two, or three modules of AAC were fused to the DHFR, also different parts of the first module were fused to the DHFR. First, the different constructs were imported and localized into wild-type (wt) mitochondria. We showed that, except for the first 30 residues of AAC, all the fusions were able to target the DHFR into the mitochondria. To our surprise, all the short constructs localize in the matrix, the longer construct of four transmembrane domains localizes half in the matrix half in the IM, and, as expected, the full-length AAC–DHFR localizes in the IM as the endogenous protein. The investigation of the machinery used by these constructs revealed that they are using the TIM23 complex.

Abbreviations used: $\Delta\Psi$, membrane potential; AAC, ADP/ATP carrier; DHFR, dihydrofolate reductase; IM, inner membrane; IMS, intermembrane space; Mx, soluble membrane proteins after sodium carbonate treatment; Mb, membrane proteins remaining in the pellet after sodium carbonate treatment; OM, outer membrane; TCA, trichloroacetic acid; wt, wild-type.

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We therefore suggest that the three modules together are required for the interaction with the TIM10 complex, which then targets the protein to the TIM22 complex and inserts it into the IM.

MATERIALS AND METHODS

Import experiments

³⁵S-labelled protein (10 µl) were added to 100 µg of purified mitochondria in import buffer (50 mM Hepes, pH 7.1/0.6 M sorbitol/2 mM KH₂PO₄/50 mM KCl/10 mM MgCl₂/5 mM L-methionine/1 mg/ml fatty-acid-free BSA) in the presence of 5 mM NADH or 5 µg/ml Valinomycin. Proteins were imported for 10 min at 30 °C. Mitochondria were harvested by centrifugation and incubated with 100 µl of breaking buffer (20 mM Hepes, pH 7.4/0.6 M sorbitol) containing 0.1 mg/ml proteinase K for 15 min on ice. proteinase K was inhibited by PMSF at 1 mM (10 min on ice). Mitochondria were then re-isolated by centrifugation and resuspended in 10 µl sample buffer and loaded on a 12 % SDS/PAGE gel. Import products were visualized by autoradiography and quantified by densitometry (FUJI Bastation, version 1.3).

Subfractionation of mitochondria and localization: mitoplasting and sodium carbonate extraction

Mitoplasting 'PK out'

After proteinase K and PMSF treatments mitochondria were harvested and then resuspended into 20 µl of breaking buffer and 180 µl of mitoplast buffer (20 mM Hepes, pH 7.4) was added. The mitoplasting was performed for 30 min on ice. Mitoplasts were then harvested (at 14 000 g) and sodium carbonate treated.

Mitoplasting 'PK in'

After import, mitochondria were harvested and then resuspended into 20 µl of breaking buffer and 180 µl of mitoplast buffer. Samples were left on ice for 15 min then 0.1 mg/ml of proteinase K was added and the sample left on ice for 15 min. Proteinase K was inhibited by PMSF at 1 mM (10 min on ice). Mitoplasts were then harvested (at 14 000 g) and sodium carbonate treated.

Sodium carbonate treatment

After mitoplasting, the supernatant corresponding to the IMS fraction was precipitated with 10 % trichloroacetic acid (TCA) for 20 min on ice. The pellet corresponding to the membranes and matrix fraction was extracted in 1 ml of 0.1 M sodium carbonate, left on ice for 30 min and spun for 20 min at 100 000 g (in Beckman TLA100.2) at 4 °C. The pellet and the supernatant (TCA precipitated) fractions were loaded onto a 12 % SDS/PAGE gel. Import products were visualized by autoradiography and quantified by densitometry (FUJI Bastation version 1.3).

Specifically for the experiment shown in Figure 2 we proceeded as follows. After import, each sample was divided into three equal aliquots ('M', 'PK out', and 'PK in'). Of these, 'M' and 'PK out' were proteinase K treated and then PMSF was added. The 'M' samples were harvested and solubilized in sample buffer: these correspond to the total import signal in intact mitochondria. The 'PK out' samples were first osmotically shocked, releasing a soluble fraction (IMS). The remaining mitoplasts were sodium carbonate treated followed by centrifugation to extract soluble and peripheral membrane proteins in the supernatant (Mx) and membrane proteins in the pellet (Mb). The 'PK in' samples were osmotically shocked after import in the presence of proteinase K where soluble IMS and peripheral IM proteins facing the IMS were degraded. Subsequent sodium carbonate treatment released

the soluble proteins ('Mx') from the membrane proteins remaining in the pellet ('Mb'). IMS and Mx samples were TCA precipitated whilst Mb samples were directly resuspended into sample buffer.

Purification of wt and *tim12-ts* mitochondria

A 10 or 20 litre fermentor with lactate medium [27a] was inoculated with 200 ml or 400 ml of a wt (D273-10B) or *tim12-ts* (CK5-8179.2) saturated preculture. Cells were grown for 16 or 32 h at 30 °C or 25 °C and shifted at 37 °C for 8 h for the *tim12-ts* strain. Cells were then harvested and mitochondria were prepared as described by Daum et al. [28] and Glick and Pon [29].

Peptide competition

The import experiment was performed using 100 µg of mitoplasts. Synthetic peptides (3 µg; Ansynt Service B. V., Roosendaal, The Netherlands) were added at the same time as the precursor. The import was performed as described above.

ATP depletion

The depletion of ATP (–ATP) was achieved by incubating the mitochondrial mix for 5 min at 30 °C prior to import with 25 µg/ml of oligomycin, 0.04 unit/µl of apyrase, 0.1 % of glycerol and 0.01 unit/µl of glycerokinase followed by another incubation of 5 min at 30 °C in presence of 0.1 µg/µl of atractyloside. The mitochondrial mix in the +ATP samples were treated as usual. All precursors (+/– ATP samples) were then supplemented with 2 mM ATP just before import to re-establish the ATP outside.

RESULTS

Constructs and import

The yeast *S. cerevisiae* contains three homologous AACs. All three of them have similar import behaviour (S. Agius and K. Tokatlidis, unpublished results). The full-length yeast AAC protein is a six transmembrane (TM1-6) segments protein of about 300 amino acids. The structure of the protein can be divided into three related repeat modules formed from two transmembrane domains joined by a loop facing the matrix. Experiments addressing targeting of AAC to the OM have shown that: (i) each repeat module can cross the OM separately [14], and (ii) it cooperates with the other two modules at the *cis* side of the OM by interacting with the Tom70 receptor [7]. To address what happens after translocation across the OM, we have engineered different deletions of AAC2 (the most abundant of the three AAC isoforms in yeast) fused to DHFR. Specifically, the fusion proteins contain one, two, four or all six TM AAC fused C-terminally to DHFR. The exact length of each construct is shown in Figure 1(A).

The constructs were first checked for their ability to transcribe and translate in an *in vitro* coupled transcription/translation system. As shown in Figure 1(B) (lane labelled '10 %') all constructs could be translated giving the respective full-length product (p). Some additional minor products of lower molecular weight could also be seen (p'), presumably arising from either internal transcription initiation sites or from degradation of the newly made precursor. All constructs were import competent and localized in a protease protected location in energized wt mitochondria (lane 'M'), with the exception of the first 30 residues which could not target DHFR to mitochondria (Figure 1B, '1-30-DHFR'). For some precursors the main import product was not the full-length protein. This is especially true for TM1-DHFR and TM5+6-DHFR. The simplest explanation would be a more efficient import

Figure 1 All fusion proteins except 1-30-DHFR are efficiently imported into mitochondria

(A) The AAC fragments are represented by white boxes and the DHFR moiety by black boxes. The amino-acid length of the AAC fragments is indicated. (B) Import of the different fusion proteins into isolated mitochondria. Proteins were synthesized in a rabbit reticulocyte lysate in the presence of [35 S]methionine. Radiolabelled proteins (10 μ l) were imported into 100 μ g of purified, energized mitochondria for 10 min at 30 °C. After import, mitochondria were re-isolated and treated with 0.1 mg/ml of proteinase K for 15 min on ice in the presence (Trit) or absence (M) of Triton-X100. Proteinase K was inhibited with 1 mM of PMSF for 10 min on ice. Triton samples were then precipitated with TCA and the pellets were solubilized in 10 μ l of sample buffer. Mitochondria were re-isolated and resuspended in 10 μ l of sample buffer. Samples were separated by SDS/PAGE (12% gel) and analysed using a phosphorimager. 10%, 10% of the input 35 S-labelled protein used in the assay; p, full-length precursor; p', secondary translation products.

of the smaller products present in the lysate. However, it is also possible that the full-length constructs are imported and, following translocation, a cleavage by a mitochondrial protease could occur. At this stage it is not possible to distinguish between these two possibilities. However, given that the full-length fusion proteins are clearly competent for import, it was decided to next ascertain their intramitochondrial localization. The simplest conclusion resulting from this experiment is that the first 30 amino acids of AAC are not sufficient to promote the targeting of the DHFR moiety into mitochondria, in contrast to the other AAC segments.

Localization: single and double transmembrane domains target the precursor to the matrix

We then wanted to investigate the precise intramitochondrial localization of all the import-competent fusion proteins. As the physiological localization of wt AAC is at the IM, we expected

the fusion proteins to localize either in the IM or in the IMS, if insufficient information is present in the particular AAC segment. To address this point, a subfractionation experiment was carried out (Figure 2, for details see Materials and methods). As a control, we localized under the same import conditions the matrix-targeted precursor Su9-DHFR and an IM protein AAC1. As expected Su9-DHFR was localized in the matrix and AAC1 in the IM (results not shown). Moreover, for each sub-fractionation the localization of endogenous marker proteins was tested (OM porin, IMS cytochrome *b2*, and IM Tim23, shown in Figure 2A).

A second control to investigate the effect of the DHFR subunit on AAC2, was to localize the AAC2-DHFR precursor. As shown in Figure 2(B), the AAC2-DHFR localizes in the Mb and Mx fractions of the 'PK out' treatment (Figure 2B, lanes 4 and 5). However, in the 'PK in' treatment the precursor disappears from the Mx fraction (Figure 2B, lane 8). This indicates that the protein in lane 5 was in fact localized in the IMS peripherally attached to the IM. Also in the 'PK in' treatment the full-length AAC2-DHFR disappears from the Mb fraction (Figure 2B, lane 7). However, a product of the size of the AAC2 is found at around 30 kDa corresponding to the expected size of the AAC2 once the DHFR is cleaved off by proteinase K. This indicates that the AAC2-DHFR localized properly in the IM (Figure 2, lanes 7) although its insertion seems to be slower than the wt AAC, as some product was found in lane 5.

Surprisingly, we found that precursors containing either one or two TM domains localize largely in the matrix, just like the one containing the loop region of AAC2 (Figure 2C, Mx lanes 5 and 8). However, the construct containing four TM domains (TM-3-4-5-6-DHFR) localizes half in the matrix and half in the IM (Figure 2C, lanes 4, 5, 7 and 8). This rather unexpected matrix localization raised the question of which TIM machinery is used by these constructs to reach the matrix.

The TIM10 complex is dispensable for the targeting to the matrix

First we searched for a putative dependence of the translocation of these matrix-targeted precursors on the TIM10 complex. The full-length AAC was shown previously to be largely impaired in its insertion at the IM when imported into mitoplasts [30]. We therefore imported the fusions into mitochondria and into mitoplasts and assessed the protease-resistance localization of matrix proteins.

As shown in Figure 3(A), the full-length AAC1 presented to mitoplasts did not insert in the IM (lower panel, lane 3), as expected. In contrast, the matrix-targeted Su9-DHFR, which does not require the TIM10 complex for its translocation, was indeed imported into the matrix where it was protected against protease (upper panel, lane 3). The fusion proteins followed the same behaviour as Su9-DHFR (Figures 3B and 3C). This indicates that the absence of the TIM10 complex did not affect the matrix localization of the AAC2-DHFR precursors.

The TIM22 complex is not required for the translocation of the fusion proteins

We then investigated whether the TIM22 complex, which is used by the full-length AAC, was also used by the fusion proteins to reach the matrix. To this end we used a temperature-sensitive strain, *tim12-ts*, which does not contain Tim12 or Tim22 and hence is deficient in the TIM22 insertion pathway. We compared translocation into *tim12-ts* and wt mitochondria and also looked at the resistance of imported precursors against proteinase K in mitoplasts. As shown in Figure 4(A), the full-length AAC1 is imported into wt mitochondria (lower panel, lane 2) and gives a lower fragment (corresponding to AAC stage V) in mitoplasts treated with

To test if the TIM23 complex is involved in this targeting process we performed a competition experiment using synthetic presequence peptides that specifically interact with the Tim23 pore-forming subunit of the TIM23 complex. As a positive competitor of the TIM23 import an Hsp60 presequence was used. As a control, we used a peptide that has the same overall charge as the Hsp60 presequence but is not capable of functioning as a mitochondrial presequence ('Synb2', [31]). The experiment was carried out on mitoplasts to avoid competition and indirect effects at the level of the TOM channel. The import of Su9-DHFR was completely abolished in the presence of the Hsp60 peptide and remained unaffected in the presence of the Synb2 peptide (Figure 5A, lanes 4 and 5). The import of the fusion proteins is greatly reduced in the presence of the Hsp60 peptide as shown in Figures 5(B) and 5(C), lanes 4, but not in the presence of buffer or Synb2 peptide (lanes 3 and 5). This is in agreement with the previous results in Figures 2 and 3 and in line with the notion that these fusion proteins behave like a matrix-targeted precursor. Interestingly, the magnitude of the competition effect decreases as the size of the fusion (AAC segment) increases. This also correlates with the relocation from the matrix to the membrane fraction. These results demonstrate that the TIM23 machinery mediates the import of these constructs, as their translocation is competed in the presence of peptides blocking specifically the Tim23 channel.

re-isolated and treated with 0.1 mg/ml of proteinase K for 15 min on ice. Proteinase K was inhibited with 1 mM of PMSF for 10 min on ice. Mitochondria were re-isolated and resuspended in $10 \mu\text{l}$ of $2 \times$ sample buffer. Lanes 3, 4, 5, after import mitochondria were proteinase K and PMSF treated. After re-isolation mitochondria were osmotically shocked and centrifuged. The supernatant corresponding to the IMS fraction was precipitated with TCA (lanes 3) and the pellet was sodium carbonate extracted. The sodium carbonate sample was centrifuged: pellet corresponding to the membrane fraction was resuspended in sample buffer (lanes 4) and supernatant corresponding to the matrix fraction was precipitated with TCA (lanes 5). Lanes 6, 7, 8: The same subfractionation as in lanes 3, 4, 5 with the exception that proteinase K treatment was made during the osmotic shock, followed by addition of PMSF. Samples were analysed by SDS/PAGE (12 % gel) and using a phosphorimager. p, full-length precursor; p', secondary translation products.

(A) Western blotting of a mitochondrial subfractionation using antibodies against cytochrome *b2* (cyto *b2*), porin and Tim23. **(B)** Import and subfractionation using ³⁵S-labelled AAC-DHFR. The black dot indicates the AAC stage V fully inserted form. **(C)** as in **(B)**, but the precursors used were the different deletions of AAC fused to DHFR as indicated. Proteins were synthesized in a rabbit reticulocyte lysate in presence of [³⁵S]methionine. 10 μl of radiolabelled protein were imported into 100 μg of purified energized mitochondria for 10 min at 30°C. Lanes 1, 10% of the input *in vitro* translated precursor. Lanes 2, intact mitochondria after import were

Figure 3 Import of all fusion proteins into mitoplasts is similar to import into intact mitochondria

(A) Control imports for Su9-DHFR (matrix-targeted) and AAC1 (IM-inserted). (B) Imports for the constructs TM1-DHFR, TM2-DHFR and Loop1-DHFR containing segments of only the first AAC2 repeat. (C) Imports of the longer constructs TM1 + 2-DHFR, TM5 + 6-DHFR and TM3 + 4 + 5 + 6-DHFR. Import was performed as in Figure 1(B) in intact mitochondria (M) or into mitoplasts (MP). After import, proteinase K treatment was done followed by PMSF. Mitochondria and mitoplasts were analysed by SDS/PAGE (12%) followed by autoradiography. p, full-length precursor; p', secondary translation products; m, mature form; i, intermediate form.

Figure 4 Fusion proteins retain their capacity to import into mitochondria from a *tim12-ts* strain

(A) Import of control proteins Su9-DHFR and AAC1. (B) Import of full-length AAC2 fused to DHFR (AAC-DHFR). (C) Import of segments of the first repeat of AAC fused to DHFR (TM1-DHFR, TM2-DHFR, loop1-DHFR). (D) Import of the longer constructs TM1 + 2-DHFR, TM5 + 6-DHFR and TM3 + 4 + 5 + 6-DHFR. Lanes 1, 10% of the input precursor. Import was into intact mitochondria using either wt mitochondria (lanes 2,3) or *tim12-ts* mitochondria (lanes 4,5). After import, proteinase K treatment was performed on mitochondria (M, lanes 2,4) or mitoplasts (MP, lanes 3,5). p, full-length precursor; p', secondary translation products; m, mature form; AAC V (indicated by a black dot), stage V of wt AAC imported form.

Figure 5 Import of fusion proteins is competed by the Hsp60 presequence peptide

Import of ^{35}S -labelled precursor was performed for 10 min at 30 °C into mitochondria (M, lanes 2) or mitoplasts (MP, lanes 3–5). Import was done either in the presence of 3 μg of synthetic peptide (Hsp60, lanes 4 or Synb2, lanes 5) or 50 mM Tris pH 8 buffer alone (buffer, lanes 3). Lanes 1, 10% of the input precursor. p, full-length precursor; p', secondary translation products; m, mature form; i, intermediate form. **(A)** Import of control protein Su9-DHFR. **(B)** Import of the first repeat of AAC fused to DHFR (TM1-DHFR, TM2-DHFR, loop1-DHFR). **(C)** Import of the longer constructs TM1 + 2-DHFR, TM5 + 6-DHFR and TM3 + 4 + 5 + 6-DHFR.

Figure 6 Import of fusion proteins is affected by matrix ATP depletion in the same manner as the matrix targeted Su9-DHFR precursor

The different fusion proteins were imported into isolated mitochondria in presence (+ATP) or absence (–ATP) of intramitochondrial ATP (see Materials and methods). After import samples were halved. The first half (M) was proteinase K treated followed by PMSF. The other half was first osmotically shocked (MP) then proteinase K and PMSF treated. Finally mitochondria (M) and mitoplasts (MP) were solubilized in sample buffer and analysed by SDS/PAGE followed by autoradiography. p, full-length precursor; p', secondary translation products; i, intermediate form of the Su9-DHFR and m, mature form of the Su9-DHFR.

experiment for matrix ATP was carried out, as matrix ATP is required for the function of the Tim44/14/Hsp70 mitochondrial import motor, but not for insertion of full-length AAC in the matrix. Under matrix ATP depletion conditions, translocation across the IM was reduced in the same manner both for the control Su9-DHFR precursor and also for the fusion proteins (Figure 6)

but not for full-length AAC-DHFR. Second, import into mitochondria from a mutant Tim23 strain (*tim23-1*, kindly given by Professor N. Pfanner, Institut für Biochemie und Molekularbiologie, Universität Freiburg, Freiburg, Germany) was equally reduced for Su9-DHFR and the fusion proteins containing TM1 and loop1 but not for full-length AAC (results not shown).

All these results strongly suggest that the fusion proteins behave as the matrix targeted protein Su9-DHFR, by using the TIM23 machinery for translocation across the IM into the matrix.

DISCUSSION

We wanted to investigate the targeting mechanism of AAC after it has crossed the outer mitochondrial membrane, and in particular the cooperation of all three repeat modules in the interaction with the TIM10 complex and in committing the precursor to the carrier pathway. To this end, we used a deletion strategy whereby different AAC segments were fused to DHFR, a non-mitochondrial passenger protein. First we checked for efficient targeting across the OM: all AAC segments contained enough targeting information to direct DHFR into mitochondria, with the notable exception of the first 30 residues that are dispensable for this process. A previous study by Adrian et al. [32] had concluded that the first 115 residues of AAC had enough targeting information to import β -galactosidase into mitochondria *in vivo*. However, an *in vitro* study by Smagula and Douglas [31] using different AAC-DHFR fusions showed that the first 72 residues of AAC were not able to target DHFR whereas the first 111 could. Our results presented here show some differences in the import level of the different proteins (i.e. in Figure 1B, translocation across the OM for TM1-DHFR is more efficient than that for TM1 + 2-DHFR). These data point towards a complex targeting mechanism for the AAC molecule after it has crossed the OM, whereby different AAC segments lead to different import levels: this is probably due to the presence of several 'import stimulation signals' and/or 'import competent conformation' signals in the hydrophobic precursor.

To address this point in greater detail, we analysed the intra-mitochondrial localization of the different fusions, after they cross the OM. The subfractionation experiments showed a strong correlation between the length of the AAC segment and the capacity of the corresponding DHFR fusion to insert at the IM. Specifically, the shorter fusion proteins containing only two TM domains were targeted exclusively into the matrix, the longer constructs containing four TM domains were targeted half in the matrix and half in the IM, whilst the full-length AAC fused to DHFR was properly inserted into the IM. Previously, Endres et al. [14] also showed that each repeat module of AAC can be imported through the OM. However, these authors found the deletions of AAC they engineered to be localized to the IMS, with the exception of the last repeat module which could be inserted into the IM. This conclusion is not supported by our results. Three obvious differences between these two studies are that first our constructs are not identical in size and, as seen for the import competency, depending on the segment present the import capacity varies (for example TM1-DHFR versus TM1-2-DHFR), secondly we have used AAC segments fused to DHFR as opposed to AAC deletions without any passenger protein fused, and thirdly Endres et al. did not use the yeast *S. cerevisiae* AAC2 but rather AAC from *Neurospora crassa* that they imported into purified *S. cerevisiae* mitochondria. Although a major influence of the DHFR moiety on the targeting of the AAC fusion was not observed in the case of full-length AAC fused to DHFR, we cannot exclude subtle differences. In addition, Wiedemann et al. [7] used different modules of AAC fused to two DHFR which were then used to block the import at the OM. These authors show (by BN/PAGE and by co-immunoprecipitations) that these fusions are still in a complex containing Tom40 and Tom70 but not Tom20. Therefore the fusions to the DHFR do not artificially create a matrix signal normally recognized by Tom20.

We have then shown that components of neither the IMS nor the TIM22 complex were required for the import of the constructs into the matrix. This targeting event was instead mediated by the TIM23 machinery, which was unexpected as AAC is believed to interact specifically with the TIM10 and TIM22 complexes and is not known to associate with the TIM23 machinery. These results actually suggest that there is a strict requirement for participation of the TIM23 channel in targeting to the matrix segments of AAC despite the fact that AAC is not a physiological substrate for the TIM23 complex. They also suggest that the TIM22 complex is incapable of targeting a protein to the matrix, and it strictly operates as an IM insertion machinery. We find all the constructs with two TM domains or less behave like a matrix-targeted precursor. The fact that hydrophobic substrates containing some of the TM domains of AAC can still go through the TIM23 complex is probably reminiscent of the capacity of TIM23 to recognize not only matrix-targeted soluble precursors but also the uncharged sorting signal of precursors like cytochrome *b₂* that are sorted via the stop-transfer mechanism.

We suggest that to be targeted to the IM via the TIM22 machinery the AAC needs to have its whole structure intact in an 'import-competent conformation' and not just some linear peptide segments. In a similar manner to the *cis* side of the OM (recruitment of Tom70 [7]), the three related repeat modules must cooperate with each other to: (i) efficiently recruit the TIM10 complex, and (ii) subsequently interact with the TIM22 complex for final insertion at the IM. In this study, we see a correlation between removal of TM domains and mislocalization to the matrix (but not stalling in the IMS). When two or one TM domain(s) are imported then a complete mislocalization in the matrix occurs. For these constructs, the TIM10 complex is not required for their targeting. Increasing the TM domains to four results in partial insertion

A Default carrier pathway: all three modules are necessary for proper insertion.

B Tim23-dependent recognition of cryptic matrix signals: mislocalisation to the matrix occurs when only part of the AAC sequence are present.

Scheme 1 Targeting of the AAC to the IM after crossing the OM

(A) Physiological, 'en-bloc' recognition by the TIM10 and TIM22 complexes that mediate targeting and insertion at the IM. (B) Mistargeting to the TIM23 machinery and matrix mislocalization occur when only part of the AAC sequence is present and the cryptic-matrix targeting signal(s) become functional.

into the IM; full insertion occurs when all 6 TM domains are present. The simplest possible explanation for these results is that the cryptic 'matrix-targeting signals' within each domain of AAC are simply hidden in the intact whole structure. When using fragments of AAC these matrix targeting signals become accessible for recognition by the TIM23 machinery and thus targeting to the matrix occurs. Such cryptic matrix signals have been reported before for other types of proteins [33,34]. These findings are summarized in the model for IM targeting of AAC after crossing of the OM shown in Scheme 1.

Our results also suggest that the four-TM-domains construct, that should still interact with the TIM10 complex as it contains TM3 and TM4, which are the TM segments most strongly recognized by TIM10, according to Curran et al. [35], may finally cross the IM via the TIM23 rather than the TIM22 complex. Therefore, interaction with the TIM10 complex in this case does not commit the precursor to the TIM22 pathway. In fact two recent reports [36,37] have shown that the small Tim complexes can interact even with OM β -barrel proteins, thus expanding the idea that TIM10 functions as a low affinity generic chaperone of the mitochondrial IMS [38]. Commitment to one or the other membrane for insertion is probably dependent on specific substrate sequences that are recognized by the membrane-embedded insertion machinery TIM22 in the IM, or the sorting machinery SAM in the OM.

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