Roles of Tom70 in Import of Presequence-containing Mitochondrial Proteins*§

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Mitochondrial protein traffic requires precise recognition of the mitochondrial targeting signals by the import receptors on the mitochondrial surface including a general import receptor Tom20 and a receptor for presequence-less proteins, Tom70. Here we took a proteome-wide approach of mitochondrial protein import in vitro to find a set of presequence-containing precursor proteins for recognition by Tom70. The presequences of the Tom70-dependent precursor proteins were recognized by Tom20, whereas their mature parts exhibited Tom70-dependent import when attached to the presequence of Tom70-independent precursor proteins. The mature parts of the Tom70-dependent precursor proteins have the propensity to aggregate, and the presence of the receptor domain of Tom70 prevents their aggregate formation. Therefore Tom70 plays the role of a docking site for not only cytosolic chaperones but also aggregate-prone substrates to maintain their solubility for efficient transfer to downstream components of the mitochondrial import machineries.

Eukaryotic cells consist of complex membrane-bounded compartments that are endowed with specific proteins. Among them, mitochondria are essential organelles and perform multiple functions ranging from energy generation to regulation of cell death. Mitochondria are estimated to consist of 1000–1500 different proteins, which represent 10–20% of proteins synthesized in eukaryotic cells. Because of the mitochondrial connection to several human diseases including neurodegenerative disorders and cancer, biogenesis and functional maintenance of mitochondria have gained widening interest, and efforts have been made to compile an extensive catalogue of the mitochondrial proteome (1).

The vast majority of mitochondrial proteins are encoded by the nuclear DNA, synthesized in the cytosol, and imported into the mitochondria, whereas only a handful of them are encoded by the mitochondrial DNA. Precise targeting of mitochondrial proteins requires recognition of mitochondrial targeting signals (2–4). The targeting signal for mitochondria is encoded within the protein itself. Most of the proteins that go across the outer and inner membranes to reach the matrix or inner membrane are synthesized as precursor proteins with an N-terminal cleavable presequence that contains a mitochondrial targeting signal. Many polytopic inner membrane proteins including those of the carrier protein family lack a presequence but instead have a mitochondrial targeting signal in the mature part as an internal signal.

Protein import into mitochondria is mediated by the translocators of the outer and inner mitochondrial membranes (5–8). Targeting information for mitochondria is recognized by the receptor subunits of the TOM40 complex including Tom20, Tom22, and Tom70 (4, 9, 10). Current understanding is that different types of targeting signals are recognized by different receptors. In general, targeting signals contained in the cleavable presequences are recognized by Tom20 and Tom22, and internal targeting signals of polytopic membrane proteins are recognized by Tom70 (9, 10). The structural analyses showed that the hydrophilic groove of Tom20 binds to the hydrophobic side of the eight-residue segment of the rat aldehyde dehydrogenase presequence, which appears to contain a five-residue motif as an element for Tom20 recognition, in the amphiphilic helical conformation (11–13). On the other hand, there are some cross-specializations between the two types of targeting signals and their cognate receptors. It has been reported that Tom70 recognizes, in addition to presequence-less polytopic membrane proteins, some presequence-containing precursor proteins including cytochrome c, alcohol dehydrogenase III, and the α- and β-subunits of F1-ATPase (Atp1p and Atp2p); yet the presequences did not appear to be the sole determinant of those presequence-containing precursors to Tom70 (14–16). The purified presequence-containing adrenodoxin precursor loaded onto the cytosolic chaperone mitochondrial import-stimulating factor was shown to bind to Tom70 first and then to Tom20 during its import into mitochondria in vitro (17). However, in contrast to Tom20, little is known about the recognition of the mitochondrial targeting signal by Tom70, although a peptide scan analysis showed that Tom70 binds to multiple segments throughout the polypeptide chain of noncleavable phosphate carrier (18). Tom70 was also shown to function as a docking site for cytosolic chaperones
such as Hsp70 (in yeast and mammals) and Hsp90 (mammals) to receive mitochondrial proteins (19).

In the present study, we took a proteome-wide approach of mitochondrial protein import *in vitro* to find a set of substrate proteins for recognition by Tom70. Translation products of total yeast RNA were imported into isolated wild type mitochondria and those without Tom70. Comparison of the imported proteins on two-dimensional electrophoresis gels between wild type and *tom70* mitochondria allowed us to identify presequence-containing precursors affected by deletion of Tom70 systematically. Then we tried to elucidate the reason for the requirement of Tom70 for the import of the identified presequence-containing mitochondrial precursor proteins. The obtained results show that Tom70 is not required for targeting signal recognition but instead for maintenance of the solubility of the presequence-containing precursor proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Yeast Strains**—The yeast haploid strain W303-1A was used as a parental strain in this study. To construct the *tom70* strain (MNA-mas70), the chromosomal *TOM70* gene was disrupted with the *URA3* marker. MNA-mas70 was transformed with the TRPI plasmid pRS314/Tom70(WT), pRS314/Tom70(38TEV), pRS314/Tom70(98TEV), and pRS314/Tom70(246) to construct yeast strains Tom70(W), Tom70(T), Tom70(T), and Tom70(T(246), respectively. The plasmids used in this study are summarized in Table 1. Yeast cells were grown at 30 °C in lactate medium (0.3% (w/v) yeast extract, 0.1% (w/v) glucose, 0.3% (w/v) potassium acetate, 0.5% (w/v) sorbitol).

**TABLE 1**

| Plasmid                  | Vector | Relevant characteristics | Source or reference |
|--------------------------|--------|--------------------------|---------------------|
| pSu9-DHFR                | pGEM-4Z| Presequence of Su9 (1–69 aa) and DHFR (187 aa) | Ref. 30             |
| pHsp60                   | pGEM-4Z| Hsp60 precursor (1–572 aa) | This study          |
| pCox4                    | pGEM-4Z| Cox4 precursor (1–155 aa)  | Ref. 28             |
| pAco1p                   | pGEM-4Z| Aco1 precursor (1–778 aa)  | This study          |
| pAtplp                   | pGEM-4Z| Atp1 precursor (1–545 aa)  | This study          |
| pCit1p                   | pGEM-4Z| Cit1 precursor (1–479 aa)  | This study          |
| pDld2p                   | pGEM-4Z| Dld2 precursor (1–530 aa)  | This study          |
| pldh1p                   | pGEM-4Z| Idh1 precursor (1–360 aa)  | This study          |
| pAtplp-Tom20             | pRS314 | Expression of wild type Tom20 under the control of own promoter | Ref. 10             |
| pAco1p-Tom20             | pRS314 | Expression of wild type Tom20 under the control of own promoter | Ref. 10             |
| pAco1p-Tom20             | pRS314 | Expression of wild type Tom20 under the control of own promoter | Ref. 10             |

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**Plasmids and Yeast Strains**—The yeast haploid strain W303-1A was used as a parental strain in this study. To construct the *tom70* strain (MNA-mas70), the chromosomal *TOM70* gene was disrupted with the *URA3* marker. MNA-mas70 was transformed with the TRPI plasmid pRS314/Tom70(WT), pRS314/Tom70(38TEV), pRS314/Tom70(98TEV), and pRS314/Tom70(246) to construct yeast strains Tom70(W), Tom70(T), Tom70(T), and Tom70(T(246), respectively. The plasmids used in this study are summarized in Table 1. Yeast cells were grown at 30 °C in lactate medium (0.3% (w/v) yeast extract, 0.1% (w/v) glu-
case, 0.05% (w/v) NaCl, 0.06% (w/v) MgCl$_2$, 0.1% (w/v) KH$_2$PO$_4$, 0.1% (w/v) NH$_4$Cl, 2% (w/v) lactic acid, pH 5.6) or SCi medium (0.67% (v/v) yeast nitrogen base without amino acids, 0.5% (v/v) casamino acids, 2% (w/v) lactic acid, 0.05% (w/v) glucose, pH 5.6).

**Two-dimensional Gel Electrophoresis—Isoelectric focusing (IEF)** for the first dimension was performed using Immobiline DryStrip (GE Healthcare Biosciences) and the IPGphor IEF system (GE Healthcare Biosciences). After import reaction, mitochondria were reisolated and solubilized with rehydration buffer (0.5% IPG buffer (GE Healthcare Biosciences), 2% CHAPS, 7 M urea, 2 M thiourea, 20 mM dithiothreitol, 1% SB3–), and the solubilized proteins were applied to Immobiline DryStrip (18 cm) rehydrated with 350 µl of rehydration buffer.

IEF was conducted on the IPGphor with a current limit of 50 µA/strip at 20 °C with the following focusing program: a gradient to 500 V for 5 min, a gradient to 4000 V for 85 min, and a gradient to 8000 V for 5 h (pI 4–7) or 10 h (pI 6–9). The strips were then treated with equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) for 15 min and subjected to SDS–PAGE for the second dimension.

**Import Assays**—Mitochondria were isolated from W303-1A (WT) and MNA-mas70 (tom70Δ) strains grown in lactate medium at 30 °C. Radiolabeled precursor proteins were synthesized with rabbit reticulocyte lysate by coupled transcription/translation in the presence of [35S]methionine. WT or tom70Δ mitochondria at 25 °C for 5 min. After the import reaction, the mitochondria were reisolated by centrifugation, and solubilized proteins were separated by two-dimensional gel electrophoresis. The mixture of radiolabeled proteins was then subjected to in vitro import analyses with mitochondria from WT cells and those from tom70-deletion mutant cells (tom70Δ). To analyze the roles of receptors by mitochondrial import assays, experimental conditions should be optimized to make the receptor-mediated step-rate-limiting in the import process (10, 20). We thus lowered the concentrations of mitochondria to the range that rendered the import rate of ADP/ATP carrier (AAC), a presequence-less substrate for the Tom70 recognition, dependent on the concentrations of mitochondria. Under this condition, import of AAC into tom70Δ mitochondria was retarded as compared with that into WT mitochondria (data not shown). We adopted this condition of mitochondrial concentrations (=0.25 mg of protein/ml) for the import of the translation products of total yeast RNA into WT and tom70Δ mitochondria at 25 °C for 5 min. After the import reaction, the mitochondria were treated with protease to remove proteins outside mitochondria, reisolated by centrifugation, and solubilized with 2% CHAPS, 7 M urea, and 2 M thiourea. The protease-protected, presumably imported proteins were separated by two-dimensional gel electrophoresis with denaturing IEF in the first dimension and SDS–PAGE in the second dimension and were detected by radioimaging (Fig. 1A). When valinomycin, which dissipates the membrane potential (ΔΨ) across the inner membrane, was added before incubation of the translation products with mitochondria, most of the spots disappeared, suggesting that they represent proteins that require ΔΨ for import (data not shown).

In total, as many as 114 spots of the imported proteins were resolved by two-dimensional gel electrophoresis under four different conditions (supplemental Fig. S1). When we compare the two-dimensional gel electrophoresis patterns of imported proteins between WT mitochondria and tom70Δ mitochondria, we find several spots exhibiting lower intensities with tom70Δ mitochondria than with WT mitochondria. Four typical examples, spots 3, 9, 13, and 22, are shown in Fig. 1A. The resolved 114 spots were thus quantified and compared between WT mitochondria and tom70Δ mitochondria (Fig. 1B). Intensities of spots 3, 6, 9, 13, 22, 30, 41, 67, 71, 72, 73, 82, 83, 90, 91, 102, and 109 for tom70Δ mitochondria were <75% of those for WT mitochondria. These spots represent proteins whose

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**Chaperone Assays for Tom70Δ**—The cytosolic domain (residues 39–617) of Tom70 with the C-terminal His$_{10}$ tag (Tom70d) was overexpressed in *Escherichia coli* strain BL21 (DE3) using the vector pET-16b (Novagen), and purified by nickel-nitrioltriacetic acid and Mono-Q 5/50 columns. Radio-labeled proteins were synthesized with PURESYSTEM by coupled transcription/translation in the presence of [35S]methionine at 37 °C for 1 h. The reaction mixture contained no additional protein, 5 µM Tom70d, or 5 µM BSA. The translation products were centrifuged at 20,000 × g for 20 min at 4 °C.

**RESULTS**

**Proteome-wide Import Analyses Reveal Substrates for Tom70**—To perform a global search for substrate proteins for Tom70 recognition in mitochondrial protein import, we isolated total RNA from wild type yeast cells, which was used for *in vitro* translation in the presence of [35S]methionine at 37 °C for 1 h. The reaction mixture contained no additional protein, 5 µM Tom70d, or 5 µM BSA. The translation products were centrifuged at 20,000 × g for 20 min at 4 °C.
Tom70 in Mitochondrial Protein Import

A

B

Imported Protein (tom70Δ/WT)

Spot Number

Spot Number

Spot Number
import into mitochondria was strongly affected by deletion of the TOM70 gene. Interestingly, some proteins corresponding to spots 21, 74, and 86 were more efficiently (> 2-fold) imported into \textit{tom70}\(\Delta\) mitochondria than into WT mitochondria. We confirmed that the levels of Tom20, Tom40, or Tom71, a minor isoform of Tom70, did not change in \textit{tom70}\(\Delta\) mitochondria (supplemental Fig. S2). Perhaps an unknown adaptation may have been caused by deletion of the TOM70 gene as previously observed for deletion of Tom20 (21). This point will be discussed later.

For identification of the spots in the two-dimensional gel electrophoresis radioimaging, we compared them with those in the two-dimensional gel electrophoresis maps for proteins associated with isolated yeast mitochondria,\(^6\) where \(\sim 90\) different protein spots were assigned to specific yeast proteins by in-gel trypsin digestion followed by mass spectrometry measurements. By referring to even this limited information on the two-dimensional gel electrophoresis maps, we could find possible candidates for the proteins whose import appeared to depend on Tom70. We thus tentatively assigned spots 3, 6, 9, 13, 67, 73, 82, and 102 to specific mitochondrial proteins, Atp2p (F\(_1\)-ATPase \(\beta\)-subunit), Tom40, Ssc1p (mitochondrial Hsp70), Dld2p (\(\beta\)-lactase dehydrogenase), Cit1p (citrate synthase), Atp1p (F\(_1\)-ATPase \(\alpha\)-subunit), Aco1 (mitochondrial aconitase), and Idh1 (isocitrate dehydrogenase), respectively. Among these eight proteins, only Atp1p and Atp2p were previously reported to depend on Tom70 for their import into mitochondria. For the numbers of the spots, see supplemental Fig. S1. The intensities for WT were set to 1.0. The error bars represent S.D. values from three independent experiments.

\(\textit{Tom70-dependent Precursors Contain Targeting Signals in the Presequences—}\) Does Tom70 recognize the presequence or mature part of the identified Tom70-dependent presequence-containing precursor proteins (\(pX\), where \(pX\) is the full-length precursor to protein \(X\))? To address this question, we tested the Tom70 dependence in the import of the fusion proteins (\(pX\)(5)-DHFR) between the presequence of \(pX\) (plus five residues in the mature domain carrying the presequence cleavage site) and a nonmitochondrial protein DHFR as a passenger domain. As controls, the pX-DHFR fusion proteins consisting of the full-length precursor proteins (\(pAco1p\), \(pAtp1p\), \(pCit1p\), \(pDld2p\), \(pIdh1p\), and \(pAtp2p\)) and DHFR were imported efficiently into mitochondria (Fig. 3A). Import of \(pAco1p\)-DHFR, \(pAtp1p\)-DHFR, \(pCit1p\)-DHFR, and \(pIdh1p\)-DHFR (like \(pAco1p\), \(pAtp1p\), \(pCit1p\), and \(pIdh1p\)) depended on Tom70, whereas that of \(pDld2p\)-DHFR or \(pAtp2p\)-DHFR (like \(pDld2p\) or \(pAtp2p\)) did not depend on Tom70. Now the DHFR fusion proteins with a presequence of the Tom70-dependent precursors, \(pAco1p\), \(pAtp1p\), \(pCit1p\), and \(pIdh1p\) (\(pAco1(5)-\text{DHFR}\), \(pAtp1(5)-\text{DHFR}\), \(pCit1(5)-\text{DHFR}\), and \(pIdh1(5)-\text{DHFR}\), respectively) were hardly or inefficiently imported into mitochondria (note that the scales for \(y\) axes differ for different charts). On the other hand, \(pDld2(5)-\text{DHFR}\) and \(pAtp2(5)-\text{DHFR}\) were imported into mitochondria more efficiently than corresponding full-length fusion proteins, \(pDld2p\)-DHFR and \(pAtp2p\)-DHFR (Fig. 3B). The impaired import of \(pAco1(5)-\text{DHFR}\), \(pAtp1(5)-\text{DHFR}\), \(pCit1(5)-\text{DHFR}\), and \(pIdh1(5)-\text{DHFR}\) was not due to aggregate formation or sequestering of the presequence in the DHFR part (supplemental Fig. S3, A and B). The mature parts of \(pAco1p\), \(pAtp1p\), \(pCit1p\), or \(pIdh1p\) lacking the presequence were not imported into WT or \(tom70\Delta\) mitochondria at all (data not shown).

Because tight folding of the DHFR domain may well hamper efficient import into mitochondria, we tested the import of fusion proteins containing the mature domains of authentic

\(^6\)K. Terao, H. Yamamoto, M. Uchida, S. Kitamura, S. Nishikawa, and T. Endo, unpublished results.

**FIGURE 1.** \textit{In vitro} import of total RNA translation products into WT and \textit{tom70}\(\Delta\) mitochondria. A, total RNA was isolated from W303-1A cells and translated with reticulocyte lysate in the presence of [\(^{35}\)S]Met, and radiolabeled proteins were imported into WT mitochondria and \textit{tom70}\(\Delta\) mitochondria. As a control, \(pSs9\)-DHFR, a fusion protein between the presequence of subunit 9 of Neurospora crassa \(F_{\mathrm{p}}\)-ATPase (\(pSs9\)) and mouse DHFR, which does not require Tom70 for import, was imported with similar or slightly higher efficiency into \textit{tom70}\(\Delta\) mitochondria than WT mitochondria (Fig. 2A). On the other hand, precursors of Aco1p (\(pAco1p\)), Atp1p (\(pAtp1p\)), Cit1p (\(pCit1p\)), and Idh1p (\(pIdh1p\)) were imported into \textit{tom70}\(\Delta\) mitochondria with lower efficiency than into WT mitochondria, whereas import rates of precursors to Atp2p (\(pAtp2p\)) and Dld2p (\(pDld2p\)) into \textit{tom70}\(\Delta\) mitochondria were similar to those imported into WT mitochondria (Fig. 2B). The different Tom70 dependence between \(pAtp1p\) and \(pAtp2p\) may be due to the difference in the presequence lengths or properties (e.g. isoelectric points) of the mature domains. Therefore we judged that at least four proteins, \(pAco1p\), \(pAtp1p\), \(pCit1p\), and \(pIdh1p\), are presequence-containing mitochondrial precursor proteins that require Tom70 for their import into mitochondria.
mitochondrial precursor proteins of Cox4p (pCox4p, cytochrome oxidase subunit IV) and Hsp60 (pHsp60), which are more susceptible to protease digestion than DHFR (supplemental Fig. S4). Although pX(5)-Cox4p fusion proteins with the presequence of pAco1p, pAtp1p, pCit1p, or pldh1p were still hardly or not imported into mitochondria (supplemental Fig. S5), the fusion proteins pX(5)-Hsp60 with any of the six presequences (pAco1(5)-Hsp60, pAtp1(5)-Hsp60, pCit1(5)-Hsp60, pDld2(5)-Hsp60, pldh1(5)-Hsp60, and pAtp2(5)-Hsp60) were imported into mitochondria (Fig. 4A), suggesting that the presequences of pAco1p, pAtp1p, pCit1p, pDld2p, pldh1p, and pAtp2p have sufficient information to target passenger proteins to mitochondria. The low ability of those presequences to direct tightly folded DHFR domain to mitochondria is likely due to their short lengths (23, 42, 37, 44, 29, and 32 residues long for pAco1p, pAtp1p, pCit1p, pDld2p, pldh1p, and pAtp2p, respectively), because presequences shorter than ~70 residues cannot engage with mitochondrial Hsp70 in the matrix without prior unfolding of the mature domains (22, 23). On the other hand, because the N-terminal 69 residues of the mature part of pHsp60 appear protease-susceptible and -labile (supplemental Fig. S4), even the short presequences (23–44 residues) can reach the matrix without unfolding of the core part of the mature domain of pHsp60, so that import efficiency of pX(5)-Hsp60 became high. The different import efficiencies between the pX(5)-Cox4p fusion proteins and pX(5)-Hsp60 fusion proteins may reflect a difference in the folding status between Cox4p and Hsp60p, as indicated by the moderate protease resistance of pCox4p as compared with highly protease-sensitive pHsp60 (supplemental Fig. S4).

It is to be noted that the pX(5)-Hsp60 fusion proteins tend to be imported into tom70Δ mitochondria more efficiently than WT mitochondria.

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### Table A

| pSu9-DHFR | WT | tom70Δ |
|-----------|----|--------|
| 10% | 2 | 5 | 10 | 2 | 5 | 10 (min) |
| p | | | | | | |
| m | | | | | | |

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### Table B

| pX | presequence | mature part |
|----|-------------|-------------|
| pAco1p | WT | tom70Δ |
| pAtp1p | WT | tom70Δ |
| pCit1p | WT | tom70Δ |
| pDld2p | WT | tom70Δ |
| pldh1p | WT | tom70Δ |
| pAtp2p | WT | tom70Δ |

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### Diagram

**A**

**B**

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Tom70 in Mitochondrial Protein Import
chondria (Fig. 4A). This unexpected observation could reflect secondary effects or adaptation caused by deletion of the TOM70 gene because some mitochondrial proteins were imported into tom70Δ mitochondria more efficiently than into WT mitochondria in our initial screening as well (Fig. 1B). To test this interpretation, we took the approach with TEV protease (TEVp) to remove the receptor domain after isolation of mitochondria (10). Briefly, we introduced a cleavage site specific for TEVp between the N-terminal transmembrane segment and the following C-terminal receptor domain of Tom70 (Tom70T mitochondria), so that we could delete the cytosolic receptor domain of Tom70 in vitro after isolating mitochondria by TEVp treatment. Pretreatment of Tom70T mitochondria with 76 μg/ml TEVp at 30 °C for 20 min resulted in the removal of >90% of the receptor domain of Tom70 (supplemental Fig. S6). Although deletion of the receptor domain of Tom70 after isolation of mitochondria did not affect the import of pSu9-DHFR, import of full-length pAco1p, pAtp1p, pCit1p, and pldh1p were retarded after deletion of the Tom70 receptor domain as compared with the case without TEVp treatment (supplemental Fig. S7). Now pAtp1(5)-Hsp60, pCit1(5)-Hsp60, and pIdh1(5)-Hsp60 (and pDld2(5)-Hsp60, as well) were imported into Tom70T mitochondria after TEV protease treatment with an efficiency similar or smaller to that without TEV protease treatment (Fig. 4B). Therefore enhanced import rates of some precursor proteins

FIGURE 2. In vitro import of pSu9-DHFR and pX into WT and tom70Δ mitochondria. Radiolabeled pSu9-DHFR (A) or radiolabeled precursor proteins pAco1p, pAtp1p, pCit1p, pldh1p, and pAtp2p (B) were incubated with wild type (WT, open circles) or tom70Δ (filled circles) mitochondria (0.1 mg of protein/ml) and analyzed as in Fig. 2. The amounts of radiolabeled proteins added to each reaction are set to 100%. p, precursor forms; m, mature forms; 10%, 10% of the input; −ΔΨ, valinomycin was added.

FIGURE 3. In vitro import of pX-DHFR and pX(5)-DHFR into wild type and tom70Δ mitochondria. A, radiolabeled fusion proteins pAco1p-DHFR, pAtp1p-DHFR, pCit1p-DHFR, pDld2p-DHFR, pidh1p-DHFR, and pAtp2p-DHFR were imported into wild type (WT) or tom70Δ mitochondria (0.1 mg of protein/ml) and analyzed as in Fig. 2. B, radiolabeled fusion proteins pAco1(5)-DHFR, pAtp1(5)-DHFR, pCit1(5)-DHFR, pDld2(5)-DHFR, pidh1(5)-DHFR, and pAtp2(5)-DHFR were imported into wild type (WT) or tom70Δ mitochondria (0.1 mg of protein/ml) and analyzed as in Fig. 2. The amounts of radiolabeled proteins added to each reaction are set to 100%. p, precursor forms; m, mature forms; 10%, 10% of the input; −ΔΨ, valinomycin was added.
into $\text{tom70}^{\Delta}$ mitochondria are most likely due to adaptation of the cells to compensate for the effects of depletion of Tom70.

Although the Presequence Is Recognized by Tom20, the Mature Part Is Responsible for Tom70 Dependence in Mitochondrial Import—If Tom70 is dispensable for the import of pX(5)-Hsp60, whose presequences pX(5) are derived from the Tom70-dependent precursor proteins, does Tom20 recognize those presequences? To address this question, we utilized Tom20 with a TEVp cleavage site between the N-terminal transmembrane segment and the C-terminal receptor domain of Tom20 (Tom20T), which allows us to analyze the effects of deletion of the Tom20 receptor domain as in the case of Tom70 (10).

Import of pHsp60 into Tom20T mitochondria after TEVp treatment was retarded compared with Tom20T mitochondria without TEVp treatment (Fig. 5A). The import of pAco1p, pAtp1p, pCit1p, pldh1p, and pAtp2p was also retarded by TEV protease treatment of Tom20T mitochondria (Fig. 5B), indicating that the targeting signals in the presequences of those precursor proteins are recognized by Tom20. The import of pDld2p was not markedly dependent on the TEVp treatment, perhaps because of its significantly low import efficiency.

Evidently, the presequences of the Tom70-dependent precursor proteins contain mitochondrial targeting signals that are recognized by Tom20. Then which is responsible for the Tom70 dependence in mitochondrial import, the presequence or mature part? To answer this question, we tested the import of the fusion proteins consisting of the presequence of Tom70-independent precursor proteins followed by the mature domain of the Tom70-dependent precursors, pAco1p, pAtp1p, pCit1p, and pldh1p into WT and $\text{tom70}^{\Delta}$ mitochondria. We chose the short (25-residue) presequence of a Tom70-independent precursor protein, pCox4p. pCox4-DHFR, a fusion protein between the Cox4 presequence and DHFR, and the pCox4 precursor (pCox4p) were imported into $\text{tom70}^{\Delta}$ mito-

FIGURE 4. In vitro import of pX(5)-Hsp60 into WT and $\text{tom70}^{\Delta}$ mitochondria or into mitochondria without the receptor domain of Tom70. A, radiolabeled fusion proteins pAco1(5)-Hsp60, pAtp1(5)-Hsp60, pCit1(5)-Hsp60, pDld2(5)-Hsp60, pldh1(5)-Hsp60, and pAtp2(5)-Hsp60 were imported into wild type (WT) or $\text{tom70}^{\Delta}$ mitochondria (0.1 mg of protein/ml) and analyzed as in Fig. 2. B, radiolabeled fusion proteins pAco1(5)-Hsp60, pAtp1(5)-Hsp60, pCit1(5)-Hsp60, pDld2(5)-Hsp60, pldh1(5)-Hsp60, and pAtp2(5)-Hsp60 were incubated with TEV protease (TEVp)-untreated (open circles) or TEVp-treated (filled circles) Tom70T mitochondria (0.25 mg of protein/ml) at 25 °C for the indicated times. The mitochondria were treated with proteinase K, and the imported proteins were analyzed by SDS-PAGE and radioimaging.

The amounts of radiolabeled proteins added to each reaction are set to 100%. p, full-length proteins; m, Hsp60; 10%, 10% of the input; $\Delta V$, valinomycin was added.
efficiency of pCox4-Dld2p and pCox4-Atp2p was too low to compare import rates between WT and \( \text{tom70}\Delta \) mitochondria, probably because of tight folding of the mature parts or aggregate formation. To increase import efficiency by efficient engagement with mtHsp70 (22, 23), we made fusion proteins consisting of the long (69 residues) pSu9 presequence followed by the mature parts of pAco1p, pAtp1p, pCit1p, pDld2p, pldh1p, and pAtp2p (pSu9-Aco1p, pSu9-Atp1p, pSu9-Cit1p, pSu9-Dld2p, pSu9-Idh1p, and pSu9-Atp2p, respectively), and tested their import. All of these fusion proteins were imported into WT and \( \text{tom70}\Delta \) mitochondria with higher efficiency than the corresponding pCox4-Xp fusion proteins (supplemental Fig. S8). However, the import rates into WT mitochondria were similar to or even lower than \( \text{tom70}\Delta \) mitochondria, suggesting that adaptation caused by the \( \text{TOM70} \) deletion masked a possible decrease in import efficiency arising from the depletion of Tom70. Therefore we tested the import of those pSu9-X fusion proteins into Tom70T mitochondria after TEV protease treatment to remove the receptor domain of Tom70. Now pSu9-Aco1p, pSu9-Atp1p, pSu9-Cit1p, and pSu9-Atp2p were clearly imported into mitochondria without TEV protease treatment more efficiently than those with TEV protease treatment (Fig. 6B). It is interesting to note that pSu9-Atp2p was imported into mitochondria in a Tom70-dependent manner, whereas pAtp2p on its own did not significantly depend on Tom70 for its import. This may suggest that the import ability of the pAtp2p presequence, which is lower than that of the pSu9 presequence, may mask the Tom70 dependence under the present \textit{in vitro} import conditions. Taken together, we conclude that although different presequences affect the import efficiency of different mature domains differently, the mature parts of pAco1p, pAtp1p, pCit1p, and pldh1p (and pAtp2p) are at least partly responsible for the Tom70 dependence in their import into mitochondria.

\textit{Tom70 Increases Solubility of Aggregate-prone Mature Domains of Tom70-dependent Precursors}—Why do the mature parts of the presequence-containing pAco1p, pAtp1p, pCit1p, and pldh1p cause Tom70 dependence in their import? Because Tom70 functions as a docking site for the cytosolic chaperone Hsp70 in yeast (19), we first asked whether those Tom70-dependent precursors tend to aggregate. To address this question, we took advantage of the use of PURESYSTEM, a cell-free bacterial translation system reconstituted from purified recombinant components and ribosomes but free of any chaperones (24). Briefly we synthesized the Tom70-dependent precursor proteins \textit{in vitro} with PURESYSTEM and analyzed the solubility of the translated proteins by centrifugation. Although pCox4, which does not depend on Tom70 in its import, is soluble in the absence of chaperones, the Tom70-dependent precursor proteins, pAco1p, pAtp1p, pCit1p, and pldh1p (and pAtp2p and pDld2p, as well) form aggregates in the absence of chaperones (Fig. 7, \textit{none}). Although the tendency of the Tom70-dependent precursor proteins to form aggregates decreased with the removal of the presequence, the corresponding mature domains of pAco1p, pCit1p, pDld2p, and pldh1p still show a propensity to form aggregates (Fig. 7). Therefore those precursor proteins with aggregate-prone mature parts likely rely on the cytosolic chaperone Hsp70 to remain soluble or import-

chondria with similar efficiency or slightly higher efficiency than WT mitochondria (data not shown). The fusion proteins (pCox4-X) with the pCox4 presequence followed by the mature parts of the Tom70-dependent precursors, pAco1p, pAtp1p, pCit1p, and pldh1p (pCox4-Aco1p, pCox4-Atp1p, pCox4-Cit1p, and pCox4-Idh1p, respectively) were imported into WT mitochondria more efficiently than into \( \text{tom70}\Delta \) mitochondria despite the lack of the original presequence (Fig. 6A). Import
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We then asked whether the role of Tom70 in the import of the Tom70-dependent precursors is more than a docking site for the cytosolic chaperones. We thus tested the solubility of those precursor proteins synthesized with PURESYSTEM in the presence or absence of the recombinant cytosolic domain of Tom70 (Tom70d) or BSA. The presence of the cytosolic domain of Tom70, but not BSA, increased the amounts of the soluble forms (Fig. 7, BSA and Tom70) in a dose (Tom70d)-dependent manner (supplemental Fig. S9). Even the mature domains, mAco1p, mMAtp1p, mDld2p, mIdh1p, and mAtp2p, became more soluble in the presence of Tom70d than BSA (Fig. 7). As a control, AAC, a highly aggregate-prone presequence-less, but Tom70-dependent membrane protein, exhibited increased solubility in the presence of Tom70d as well, whereas aggregate formation of phosphate carrier, another presequence-less aggregate-prone carrier protein, was not relieved by Tom70d (Fig. 7). Therefore, the cytosolic domain of Tom70 has a chaperone-like function to maintain solubility of many, if not all, aggregate-prone substrate proteins.

DISCUSSION

The mitochondrial outer membrane translocator, the TOM40 complex, contains at least three import receptors, Tom20 and Tom22 for presequence-containing precursor proteins and Tom70 for presequence-less proteins. Nevertheless, previous observations that some presequence-containing precursor proteins also depend on Tom70 for their import had been left out of further investigations. In the present study, we took a proteome-wide approach of mitochondrial protein import in vitro and identified several new presequence-containing proteins (mAco1p, mAtp1p, mCit1p, mDld2p, mIdh1p,
and pAtp2p) that require Tom70 for their import into mitochondria. By testing their in vitro import into WT and tom70Δ mitochondria, we found that pAco1p, pAtp1p, pCit1p, and pIdh1p indeed depended on Tom70 for their import. Tom70 dependence in import was found for only the pSu9 fusion protein of the mature part of pAtp2p, but not pDld2p. Therefore, identification of the presequence-containing Tom70 substrates by in vitro import of total RNA translation products followed by two-dimensional gel analyses was confirmed by individual in vitro import for pAco1p, pAtp1p, pCit1p, and pldh1p (and pAtp2p), but not for pDld2p. The spot for Dld2p in two-dimensional gel analyses (spot 3 in Fig. 1A) may be overlapped by other proteins. We then performed systematic analyses of in vitro import of fusion proteins containing various combinations of the presequences or mature parts of Tom70-independent precursor proteins or nonmitochondrial DHFR and the
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presquences, mature parts, or full-length forms of the identified Tom70-dependent precursor proteins. We thus found that the presquences of the Tom70-dependent precursor proteins require the general presquence receptor Tom20 for their import, whereas the mature parts are responsible for the Tom70 dependence for their import. When synthesized in the reconstituted, cell-free translation system lacking cytosolic chaperones, both full-length forms and mature parts of the Tom70-dependent precursor proteins exhibited formation of insoluble aggregates, which were suppressed by the presence of the receptor domain of Tom70, suggesting that Tom70 has a chaperone-like function. Although a chaperone-like function of Tom70 was previously speculated, direct experimental evidence was lacking (15, 25).

Many mitochondrial precursor proteins tend to form aggregates because of the properties of their presquences, mature parts, or both (26). Therefore aggregate-prone mitochondrial precursor proteins require cytosolic chaperones including yeast Hsp70 (17, 27, 28) to maintain their solubility or import competence. Tom70 is known to function as a docking site for those chaperones to recruit the chaperone-substrate complexes to the TOM40 complex (19). Now the present study indicated that although Tom70 can recognize substrates with internal targeting signals as their cognate receptor, it also receives some substrates as a chaperone even if they do not have internal signals. This is in good accordance with the previous bioinformatics-based proposal that hydrophobic mitochondrial proteins, irrespective of the presence of presquences, could be substrates for Tom70 (17). Perhaps a cytosolic chaperone and its substrate may still form a complex on Tom70 for a while. Then after dissociation of the chaperone-substrate complex, Tom70 likely maintains solubility of the bound substrate protein for efficient transfer to downstream components such as Tom20, Tom22, and Tom40 of the TOM40 complex. Indeed the previous study of peptide scanning analyses showed that some of the Tom70-binding peptides of its substrate, phosphate carrier, are rather hydrophobic (18).

The crystal structure of the cytosolic domain of yeast Tom70 contains a putative binding pocket for substrate proteins, which consists of many hydrophobic residues (25, 29). It is likely that this pocket would provide a binding site for the solvent-exposed hydrophobic region of the unfolded or loosely folded substrate proteins, thereby preventing them from aggregate formation. Systematic analyses to measure the effects of amino acid replacement of the binding pocket of Tom70 on aggregate formation of newly synthesized substrate proteins in the absence of cytosolic chaperones will reveal a more detailed structural basis for the chaperone function of Tom70.

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