Human mitochondrial import receptor Tom70 functions as a monomer

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Abstract

The mitochondrial import receptor Tom70 (translocase of the mitochondrial outer membrane 70) interacts with chaperone–preprotein complexes through two domains: one that binds Hsp70 (heat-shock protein 70)/Hsc70 (heat-shock cognate 70) and Hsp90, and a second that binds preproteins. The oligomeric state of Tom70 has been controversial, with evidence for both monomeric and homodimeric forms. In the present paper, we report that the functional state of human Tom70 appears to be a monomer with mechanistic implications for its function in mitochondrial protein import. Based on analytical ultracentrifugation, cross-linking, size-exclusion chromatography and multi-angle light scattering, we found that the soluble cytosolic fragment of human Tom70 exists in equilibrium between monomer and dimer. A point mutation introduced at the predicted dimer interface increased the percentage of monomeric Tom70. Although chaperone docking to the mutant was the same as to the wild-type, the mutant was significantly more active in preprotein targeting. Cross-linking also demonstrated that the mutant formed stronger contacts with preprotein. However, cross-linking of full-length wild-type Tom70 on the mitochondrial membrane showed little evidence of homodimers. These results indicate that the Tom70 monomers are the functional form of the receptor, whereas the homodimers appear to be a minor population, and may represent an inactive state.

Keywords

chaperone; dimerization; heat-shock protein (Hsp); mitochondrion; protein structure; translocase of the mitochondrial outer membrane complex (Tom complex)

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AUTHOR CONTRIBUTION

Experiments were performed by Anna Fan (Figures 2–5 and Supplementary Figure S1) and Lisandra Gava (Figure 1). The experimental strategy was directed by Jason Young and Carlos Ramos, in consultation with Anna Fan and Lisandra Gava. The manuscript was written by Anna Fan and Jason Young, with assistance from Carlos Ramos. Figures were prepared by Anna Fan, Lisandra Gava and Carlos Ramos. The project was initiated and supervised by Jason Young.

INTRODUCTION

Mitochondria are double-membrane-bound organelles that are crucial to many physiological functions, such as cellular respiration, metabolism of lipids, amino acids and iron, and regulation of apoptosis. There are 1000–2000 proteins in mitochondria but few of them (eight in budding yeast *Saccharomyces cerevisiae*, 13 in humans) are encoded by the mitochondrial genome [1]. The vast majority of mitochondrial proteins are encoded in the nucleus, translated by cytosolic ribosomes and subsequently imported into mitochondria. The Tom (translocase of the mitochondrial outer membrane) complex includes two integral membrane import receptors, Tom20 and Tom70, and a GIP (general import pore) complex. Many proteins destined for the matrix and intermembrane space are synthesized with N-terminal amphipathic presequences, which are recognized by Tom20 via hydrophobic interactions [2–4]. In contrast, Tom70 preferentially recognizes preproteins with internal hydrophobic targeting sequences, many of which are inner membrane metabolite carriers [2,3,5]. Tom70 has also been implicated in the import of several large hydrophobic matrix proteins [6,7] and Tom40 [8], and the insertion of some outer membrane proteins [9–11]. After binding by the Tom70 or Tom20 receptors, precursor proteins are then transferred to the GIP for translocation across the outer mitochondrial membrane and sorted to their appropriate sub-compartments [2].

The Tom70-mediated import pathway is chaperone-dependent. Prior to import, Tom70-dependent preproteins are bound by Hsp (heat-shock protein) chaperones, notably Hsp70/Hsc70 (heat-shock cognate 70) and Hsp90, as well as the human (mammalian) DNAJA1 and DNAJA2 co-chaperones [12–15]. Tom70 contains an N-terminal transmembrane domain followed by a cytosolic TPR (tetratricopeptide repeat) clamp domain similar to those first structurally characterized in the cytosolic co-chaperone Hop [16]. This TPR clamp domain serves as a docking site for Hsc70 and Hsp90 and thus for the multi-chaperone complexes that contain preprotein. Preproteins are then thought to interact with Tom70 directly via a C-terminal domain separate from the TPR clamp [12,17,18]. Cross-linking experiments showed direct contacts between preproteins and chaperones, as well as contacts between preproteins and Tom70 itself [12,13]. Progression of preproteins from the Tom70 complex to the GIP is dependent on ATP and the Tim9 (translocase of the mitochondrial inner membrane 9)/Tim10 components in the intermembrane space [5,19,20]. Geldanamycin, an ATP-mimetic for Hsp90, blocks progression of preproteins from the receptor-bound state to the GIP complex, suggesting that ATP-dependent cycling by Hsp90 and, most probably, Hsc70 assists the subsequent translocation step [13]. However, the mechanism by which Tom70 co-ordinates interactions with preproteins and chaperones remains unresolved.

The oligomeric state in which Tom70 functions is controversial, based on experiments with the receptor from *S. cerevisiae*. Cross-linking of translocation intermediates of the Tom70-dependent preprotein AAC (ADP/ATP carrier) produced high-molecular-mass species consistent with binding to two Tom70 molecules [20,21]. Tom70 from isolated yeast mitochondria was also shown to generate a cross-linked dimer [19]. Targeting of AAC to the outer membrane led to the recruitment of up to three homodimers of yeast Tom70 in higher-molecular-mass complexes, by analysis on BN-PAGE (blue native PAGE) [19]. As a result of these original studies, the functional state of yeast Tom70 on the membrane was believed

to be a dimer. The crystal structure of the soluble cytosolic fragment of yeast Tom70 also suggested a homodimeric model [22], in which each molecule has a 'closed' arrangement with the TPR clamp and C-terminal domains tightly packed so that the chaperone binding sites were not readily accessible. On the contrary, biophysical studies with a similar fragment of yeast Tom70 suggested a very different conclusion. AUC (analytical ultracentrifugation) and SEC (size-exclusion chromatography) indicated that soluble yeast Tom70 was monomeric [23], and measurements of its unfolding transitions suggested that the domains within Tom70 were relatively independent of each other [23,24]. SAXS (small-angle X-ray scattering) data, when fitted to predicted profiles by various methods, also indicated a monomeric yeast Tom70 with an 'open' end-to-end arrangement of the domains, and with a small fraction in a more flexible state with its domains dissociated. Peptide ligands corresponding to Hsc70 in yeast (Ssa1) and preprotein bound to soluble Tom70 readily without changing its overall shape [18]. Most recently, the crystal structure of a close Tom70 homologue, *S. cerevisiae* Tom71, was solved. Despite more than 50% sequence identity and a similar domain arrangement with yeast Tom70, the crystal structure of yeast Tom71 depicted a monomer with an elongated conformation resembling the open model. Structures of Tom71 in complex with C-terminal peptides of yeast Hsc70 (Ssa1) and Hsp90 (Hsp82) further suggested that chaperone docking induced opening of the preprotein binding site of the monomeric receptor [25]. These conflicting models of Tom70 imply different mechanisms of function.

The mammalian homologue of Tom70 was identified by Suzuki et al. [26], but structural data are not yet available and its biophysical properties remain uncharacterized up to now. In humans, there is only one gene encoding Tom70, which is equally divergent from yeast Tom70 and Tom71. Human Tom70 shares only 24% sequence identity with yeast Tom70 and, despite having the same overall domain architecture and chaperone-dependent mechanism, it cannot substitute functionally for its yeast homologue [12]. This suggests that there are significant differences between the fungal and mammalian import systems at the functional, and perhaps even structural, level.

Here, we demonstrate that the cytosolic fragment of human Tom70 exists in equilibrium between monomer and dimer. Mutagenesis of the predicted dimerization interface shifts the equilibrium to the monomeric form and increases the level of pre-protein targeting, but not chaperone docking. Cross-linking of endogenous human Tom70 on mitochondria isolated from HeLa cells failed to generate homodimeric cross-links, although other cross-linked forms were observed. Taken together, these results show that the functional state of human Tom70 is monomeric.

EXPERIMENTAL

Materials

The expression vectors pGEM4 and pGEM4Z were purchased from Promega, pET11a and pET15b from Novagen and pPro-ExHTa from Invitrogen. The QuikChange[®] mutagenesis kit was from Stratagene, the TNT[™]-coupled reticulocyte lysate system was from Promega and [³⁵S]methionine/cysteine labelling mix was from GE Healthcare. Cross-linkers SMCC [succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate], BMH

(bismaleimido)hexane) and s-SMCC (sulfo-SMCC) were from Pierce. Unless stated otherwise, all chemical reagents were from Sigma or BioShop Canada.

Plasmids

Sequences encoding bovine PiC (phosphate carrier) and bovine OGC (oxoglutarate carrier) were in pGEM4 [27,28], rat Hsc70 was in pET11a and human Hsp90 α was in pET15b [29]. The full-length human Tom70 was in pGEM4Z and the cytosolic fragment (residues 111–608) was in pProExHTa [12]. The mutation R192A in the TPR clamp of human Tom70 was previously introduced by PCR [12] and the mutation YS585AA in the predicted dimerization interface was introduced using the QuikChange[®] mutagenesis kit.

Proteins and antibodies

The His-tagged cytosolic fragments of human Tom70 [denoted WT₁₁₀ (where WT is wild-type), YS585AA₁₁₀ and R192A₁₁₀] were purified as described previously [13]. Briefly, the proteins were expressed in *Escherichia coli* BL21(DE3) cells and induced with 0.2 mM isopropyl β -D-thiogalactoside at 37°C for 2 h. The cells were lysed by extrusion in a French press and the cell debris was removed by centrifugation. The His-tagged proteins were bound to a nickel–Sepharose high-performance column (GE Healthcare) equilibrated in 20 mM KH₂PO₄, pH 7.5, 500 mM NaCl and 20 mM imidazole, and eluted with 20 mM KH₂PO₄, pH 7.5, and 300 mM imidazole. The protein purity was assayed by SDS/PAGE (12% gels), and the yield was determined by absorbance at 280 nm. Specific rabbit polyclonal antibodies were raised against Tom70 WT₁₁₀.

Biophysical analysis

CD measurements were recorded in a JASCO J-810 spectropolarimeter, using a 1 mm pathlength cuvette and protein concentration from 1.9 to 7.8 μ M in 20 mM Tris/HCl, pH 8.5, and 150 mM NaCl. CDNN deconvolution software [30] was used to predict the secondary structure content.

SV (sedimentation velocity) and SE (sedimentation equilibrium) experiments were carried out using a Beckman Optima XL-A ultracentrifuge and an AN-60Ti rotor at 20°C [31]. Scan data acquisition were taken at 280 nm, in buffer containing 20 mM Tris/HCl, pH 8.5, 150 mM NaCl and 5 mM magnesium acetate at 9000 to 11000 rev./min and protein concentrations from 150 to 1500 μ g/ml for SE and at 30000 rev./min and protein concentrations from 500 to 1500 μ g/ml for SV experiments. SE data were analysed by nonlinear regression using the Origin software package (MicroCal) and a self-association method, applying either a monomer model (fixed molecular mass of 60 kDa) or a monomer–dimer model (fixed molecular mass of 60 kDa and N2 equals 2, which corresponds to a dimer). Protein distribution was fitted to the following equation [32]:

$$C = C_{\text{monomer}, r_0} e^{\left[\frac{M(1-V_{\text{bar}}\rho)\omega^2(r^2-r_0^2)}{2RT} \right]} + KB^*(C_{\text{monomer}, r_0})^n e^{\left[\frac{M(1-V_{\text{bar}}\rho)\omega^2(r^2-r_0^2)}{2RT} \right]}$$

where C is the protein concentration at radial position r , C_{monomer} , r_0 is the protein concentration at radial position r_0 (initial radial position), ω is the centrifugal angular velocity, K_B is the binding constant and n is the stoichiometry. The software Sednterp (<http://www.jphilo.mailway.com/download.htm>) was applied to estimate the partial specific volume at 20°C (0.7327 ml/g), buffer density and buffer viscosity. SV data were analysed with SedFit software [33] using the continuous sedimentation distribution model to yield the sedimentation coefficient and frictional ratio.

SEC-MALS (SEC and multi-angle light scattering) experiments were performed using an analytical Superdex 200 10/300 column (GE Healthcare) or BioSuite 250 4 μm UHR SEC column (Waters) equilibrated in buffer CG (20 mM Hepes/KOH, pH 7.5, 100 mM potassium acetate and 5 mM magnesium acetate), combined with online static light scattering by absorbance and differential refractive index detectors (miniDawn TREOS and Optilab rEX, Wyatt Technology). Recombinant human Tom70 WT₁₁₀ and YS585AA₁₁₀ were loaded at 2000 $\mu\text{g/ml}$ (unless otherwise indicated) with a sample size of 50 μl , and experiments were performed at room temperature (22°C). Data were analysed with the ASTRA software package (Wyatt Technology).

Cross-linking and co-precipitation

Purified proteins were incubated at 10 μM in buffer CG with 1% DMSO or 100 μM SMCC at 4°C for 15 min. The reactions were quenched with 100 mM DTT (dithiothreitol) and 10 mM ethanolamine, pH 8, at 4°C for 15 min and analysed by SDS/PAGE (8% gels) and immunoblots. For SEC, 200 μl of WT₁₁₀ was cross-linked and quenched at the above conditions and then resolved by a Superdex 200 10/300 column (GE Healthcare) equilibrated in buffer CG. Eluted protein was collected in 0.3 ml fractions, precipitated by trichloroacetic acid, and then analysed by SDS/PAGE (8% gels) and Coomassie Blue staining.

Co-precipitation with nickel–Sepharose was performed [13] with radiolabelled OGC, PiC, Hsc70 or Hsp90 at a final concentration of 5 μM WT₁₁₀, YS585AA₁₁₀ or R192A₁₁₀ as indicated, and analysed by autoradiography.

For cross-linking, the co-precipitated OGC or PiC complex was resuspended in 100 μl of buffer CG, and then incubated with 1% DMSO or 100 μM BMH at 4°C for 15 min. The reaction was quenched by 200 mM DTT and, where indicated, washed by denaturing buffer containing 250 mM NaCl, 20 mM Tris/HCl, pH 8, and 1% SDS.

Mitochondrial targeting

Mitochondria were isolated as described previously [11,13], except that HeLa cells were used. HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 4.5 g/l glucose, 36 mg/l pyruvate, 2 mM glutamine and 10% fetal bovine serum (Invitrogen). The cells were washed with PBS, collected by scraping in PBS and centrifugation, resuspended in buffer B (10 mM Hepes/KOH, pH 7.5, 220 mM mannitol, 70 mM sucrose and 1 mM EDTA) containing 2 mg/ml BSA and Complete Mini Protease Inhibitors (Roche), and lysed by seven passes through a 27-gauge needle. Mitochondria

were recovered from the postnuclear supernatant by centrifugation at 12000 *g* for 5 min and resuspended at 2 mg/ml in buffer containing 20 mM Hepes/KOH, pH 7.5, 250 mM sucrose, 80 mM potassium acetate, 5 mM magnesium acetate, 10 mM succinate, 2 mM ADP and 2 mM DTT. The isolated mitochondria were competent for import, dependent on the inner membrane potential, and potential-independent insertion into the outer membrane. The targeting of cell-free translated proteins to mitochondria was performed as described [13]. After targeting, mitochondria were resuspended in buffer B at 1 mg/ml and incubated with 1% DMSO or 1 mM s-SMCC at 4°C for 15 min. The reactions were quenched with 100 mM DTT and 10 mM ethanolamine, pH 8, at 4°C for 15 min and analysed by SDS/PAGE and autoradiography.

BN-PAGE

BN-PAGE analysis was performed as described previously [5,13]. Purified proteins (WT₁₁₀ or YS585AA₁₁₀) were resolved in buffer CG containing 1% digitonin (Calbiochem), 10% glycerol and 0.5% Coomassie Blue G-250 (EMD Chemicals). HeLa mitochondria were solubilized in buffer containing 20 mM Hepes/KOH (pH 7.5), 50 mM potassium acetate, 1% digitonin and 10% glycerol at 4°C. After a clarifying spin, Coomassie Blue G-250 was added to 0.5% for loading. Samples were loaded on to 6–16.5% gradient gels. Purified proteins were visualized by Coomassie Blue staining, for endogenous Tom70 by immunoblotting against Tom70, and for radiolabelled full-length human Tom70 (WT or YS585AA) by autoradiography. For two-dimensional PAGE, purified recombinant WT₁₁₀ and YS585AA₁₁₀ were cross-linked with 100 μ M SMCC and quenched as described, and then resolved by BN-PAGE in the presence of 1% digitonin, 10% glycerol and 0.5% Coomassie Blue G-250 as above. After that, gel strips were loaded on to SDS/PAGE (8% gels) and resolved in the presence of 1% agarose containing 5% SDS, 50 mM Tris/HCl, pH 6.8, 25 mM DTT and 5% glycerol, followed by immunoblotting against Tom70.

RESULTS

The cytosolic region (residues 111–608) of His-tagged human Tom70 (Tom70 WT₁₁₀) was purified as described previously [13]. CD spectroscopy indicated that it was natively folded and largely α -helical ($66 \pm 4\%$) by deconvolution analysis (Figure 1A), which is similar to that predicted by homology to yeast Tom70. To examine its oligomeric state in solution, we performed AUC. SV analyses (Figure 1B) were performed at concentrations from 500 μ g/ml (dashed line) to 1500 μ g/ml (solid line.) The $c(s)$ distribution of the recombinant Tom70 WT₁₁₀ yielded two species, in major and minor fractions, seen most clearly in the plot for the highest concentration. The majority had a sedimentation coefficient of 3.8 S, in agreement with the values of 3.9 S and 3.6 S observed for yeast Tom70 by Beddoe et al. [23] and Mills et al. [18] respectively. In addition, the $c(s)$ distribution of Tom70 WT₁₁₀ showed a minor peak with a sedimentation coefficient of 6.2 S containing at least 15% of total protein, indicating that some of the protein is present in a higher oligomeric state. The 3.8 S and 6.2 S sedimentation coefficients were consistent with molecular masses of approx. 60 kDa and 120 kDa respectively.

To further investigate this, SE experiments, shown in Figure 1(C), were performed at concentrations of 150 $\mu\text{g/ml}$ (left panels) and 1500 $\mu\text{g/ml}$ (right panels). Notably, concentration distributions shown by absorbance scans (Figure 1C, lower panels) could not be fitted to a single species model, as seen by the biased distribution of residuals (Figure 1C, upper panels). Instead, the concentration distributions fit best to a self-association model as shown by residuals that were evenly distributed over the regression line (Figure 1C, middle panels) with an estimated equilibrium dissociation constant of approx. 10^{-5} M. So, the SE velocity results were in agreement, suggesting that a single species model could not account for the full behaviour of the human Tom70 soluble fragment. A higher-order oligomer with a sedimentation coefficient consistent with a homodimer appeared to make up a minor, but appreciable, fraction of the population.

To address this further and study potential functional consequences, we performed site-directed mutagenesis to disrupt the putative homodimerization of Tom70. Although an earlier crystal structure by Wu and Sha [22] depicted yeast Tom70 as a compact homodimer with a dimerization interface that is almost flat, Mills et al. [18] characterized Tom70 as an elongated open monomer in solution by SAXS and proposed an alternate interpretation of the crystal structure with two intertwining molecules of the open Tom70. Therefore, we performed homology modelling of human Tom70 using Swiss-Model [34] with the homodimeric structures as templates, and the model was depicted in an open monomeric conformation by alternate pairing of N- and C-terminal domains, as proposed by Mills et al. [18] (Figure 2A). Although the SAXS-refined monomer model in the study by Mills et al. [18] was not available as a template, a homology model of open monomeric human Tom70 was also generated using the unliganded monomeric structure of yeast Tom71 [25] as a template (Supplementary Figure S1A at <http://www.BiochemJ.org/bj/429/bj4290553add.htm>). In all models, hydrophobic contacts were predicted between helices of the TPR clamp and C-terminal domains: between domains from different subunits in the dimeric model, and from the same polypeptide in the monomeric models. In particular, Tyr⁵⁸⁵ and neighbouring Ser⁵⁸⁶ interacted closely with residues Gly²³⁶, Ala²⁴⁰ and Lys²⁴³. We therefore created a double mutation, YS585AA (Figure 2A, black spheres) at the predicted domain interface and purified the mutant protein, YS585AA₁₁₀, as a recombinant fusion protein. The mutation was expected to disturb intersubunit contacts in a dimeric form of Tom70, as well as promoting a domain-dissociated and more flexible state of monomeric protein.

To confirm the effect of the mutation, the purified proteins WT₁₁₀ and YS585AA₁₁₀ were subjected to SEC followed by in-line MALS with concentration determination by refractive index (Figure 2B). Tom70 WT₁₁₀ eluted in two peaks at 12.5 ml and 14.3 ml, and the proportion of early peak to late peak was estimated to be 50:50. Although the mutant YS585AA₁₁₀ also eluted in two peaks at 12.6 ml and 14.3 ml, the proportion of early peak to late peak was significantly reduced to 30:70. The molecular masses of protein in the early peaks were unambiguously determined by MALS to be 120 kDa for both WT₁₁₀ and YS585AA₁₁₀ (119.5 kDa and 119.9 kDa respectively, rounded up), indicating that the peaks were dimeric human Tom70 species. The molecular masses of the protein in the late peaks were calculated to be 71.8 kDa for WT₁₁₀ and 66.2 kDa for YS585AA₁₁₀, which should correspond to the monomeric forms with predicted molecular masses of 59.6 kDa

and 59.5 kDa respectively. The molecular mass estimations for the late peaks were probably skewed upwards because of co-eluting dimeric species trailing from the early peaks, a common chromatographic occurrence that did not affect analysis of the early peaks. In addition, SEC analysis of WT₁₁₀ at progressively lower concentrations produced increasingly higher late peaks, in agreement with a monomer–dimer equilibrium in solution (see Supplementary Figure S1B). Although there was no evidence of different monomeric conformations between the open elongated and the domain-dissociated flexible states, the difference in physical and structural characteristics may be too small to be resolved by the SEC separation. Overall, SEC-MALS provided firm evidence that human recombinant Tom70 can exist as monomer and dimer, consistent with our AUC experiments (Figures 1B and 1C), and that the YS585AA mutation shifts the monomer–dimer equilibrium towards the monomeric form.

In addition to the biophysical methods, we also characterized the effect of the YS585AA mutation by chemical cross-linking for comparison with the earlier yeast Tom70 studies. We treated the purified proteins with the heterobifunctional cross-linking reagent SMCC, followed by immunoblotting against Tom70 (Figure 2C). SMCC is amine- and sulfhydryl-reactive and similar to the cross-linking reagent MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester) used in previous studies [19,21]. Immunoblotting following SDS/PAGE showed that both WT₁₁₀ and YS585AA₁₁₀ migrated at 60 kDa in the absence of cross-linker. In the presence of SMCC, WT₁₁₀ formed a strong homodimeric cross-link band at approx. 150 kDa (Figure 2C, XL₂), which was not observed with YS585AA₁₁₀. Instead, YS585AA₁₁₀ formed a cross-link band slightly above 60 kDa (Figure 2C, XL). We interpret this cross-link as an internal cross-link of the monomeric protein as it clearly migrates too rapidly to be any type of cross-linked dimer. It is possible that a more flexible monomeric conformation of the mutant could favour an internal cross-link, but it is highly unlikely that a more rigid WT monomer could produce the 150 kDa cross-link. We tested our result further by analysing cross-linked WT₁₁₀ by SEC (Figure 2D). The 150 kDa cross-link band eluted in a single peak between 12.3 and 13.5 ml (Figure 2D, XL₂), identical to the early peak in the SEC-MALS experiments. In contrast, the 60 kDa band spanned a wider range between 12.6 and 14.7 ml, in which both un-cross-linked dimeric Tom70 as well as monomeric Tom70 may be present. Taken together, we have clear evidence, from AUC, SEC-MALS and cross-linking, of dimeric as well as monomeric soluble human Tom70, and furthermore that our YS585AA mutant disrupts the homodimerization and shifts the equilibrium towards the monomer.

We thus proceeded to test the effect of the oligomeric state of human Tom70 on preprotein targeting and chaperone docking. Previously established co-precipitation assays were used [13], including the R192A mutation in the TPR clamp domain (Figure 2A, grey spheres) as a negative control. This mutation was shown to abolish chaperone-docking and preprotein targeting to human Tom70 [12]. The Tom70-dependent preproteins, OGC and PiC, were radiolabelled by cell-free translation and incubated with His-tagged Tom70 WT₁₁₀, YS585AA₁₁₀ or R192A₁₁₀ as indicated. Stable complexes were isolated on nickel–Sephacrose beads and analysed by SDS/PAGE and autoradiography. OGC was clearly pulled down by Tom70 WT₁₁₀ (Figure 3A, lane 3). Remarkably, YS585AA₁₁₀ increased the level of OGC recovered to approx. 140% of WT₁₁₀ (**P* = 0.039, Figure 3A, lane 4). In

contrast, the level of OGC recovered by R192A₁₁₀ was reduced practically to the negative control without Tom70 (Figure 3A, lanes 2 and 5). Pull-down of the preprotein PiC showed a similar pattern (Figure 3B), with the amount of PiC recovered by YS585AA₁₁₀ increased to approx. 120% of WT₁₁₀ (** $P = 0.0013$). The mutant was therefore more efficient at preprotein targeting than WT Tom70.

Tom70 is thought to directly contact preproteins after the chaperone-docking step. We asked whether this increase in preprotein targeting was a direct result of increased preprotein interaction with the import receptor, or an indirect effect from increased chaperone docking. We used the same co-precipitation assay except with radiolabelled Hsc70 (Figure 3C) and Hsp90 (Figure 3D) to monitor the level of chaperone docking on to Tom70 [13]. Radiolabelled chaperones Hsc70 (Figure 3C) and Hsp90 (Figure 3D) were recovered by WT₁₁₀ and YS585AA₁₁₀ to the same extent, whereas the mutant R192A₁₁₀ abolished chaperone docking to the level of the empty beads control. This indicates that our dimerization mutant, YS585AA₁₁₀, binds chaperones as efficiently as WT₁₁₀. We thus provide clear evidence that monomeric human Tom70 has higher levels of preprotein targeting via a mechanism that is separate from chaperone docking.

The oligomeric status may also affect the affinity of Tom70–preprotein interaction. Previous work using co-precipitation, cross-linking and resistance to denaturants revealed direct interactions between PiC and purified Tom70 [12]. We studied the preprotein–Tom70 contacts by co-precipitating the radiolabelled preproteins PiC and OGC with WT₁₁₀ or YS585AA₁₁₀ as above. The stably recovered complexes were then treated with a homobifunctional sulphhydryl-reactive cross-linker BMH, washed with buffer containing SDS and analysed by SDS/PAGE and autoradiography. Upon cross-linking of the isolated PiC complexes with WT₁₁₀ or YS585AA₁₁₀, three SDS-resistant bands appeared at the higher-molecular-mass range 100–200 kDa (Figure 4A). In particular, cross-linked band ‘a’ was significantly stronger in PiC–YS585AA₁₁₀ than in PiC–WT₁₁₀ complexes (Figure 4A, compare arrow XL_a, lanes 3 to 4 and 6 to 7). The resistance of the enhanced band ‘a’ to SDS confirmed that it represented a direct contact between preprotein and Tom70. Furthermore, a stronger cross-link band was also observed in OGC–YS585AA₁₁₀ complexes (Figure 4B, compare arrow XL_a, lanes 3 and 6). The appearance of the stronger cross-links suggested that the YS585AA mutation enhanced direct contacts between the preprotein and Tom70, and that preprotein–Tom70 interactions are stabilized in the monomeric form of the receptor. Taken together, the monomeric Tom70 binds preproteins, but not chaperones, more efficiently, thus increasing preprotein targeting.

We concluded from our co-precipitation and cross-linking of stable preprotein–Tom70 complexes that the functional form of human Tom70 is monomeric, which led us to investigate human Tom70 in its endogenous membrane context. In experiments with isolated yeast mitochondria, cross-linking with 1 mM MBS led to a dimeric Tom70 species [19,21]. The same result is observed with the recombinant human Tom70 using a related cross-linker SMCC (Figure 2C). Therefore, we isolated mitochondria from HeLa cells and performed cross-linking with the membrane-impermeable cross-linker, s-SMCC. Mitochondria were incubated with 1% DMSO or s-SMCC in concentrations of 1–1000 μ M and then analysed by SDS/PAGE and immunoblotting for Tom70 (Figure 5A). Surprisingly, human Tom70 did

not form a homodimeric cross-link species at any of the cross-linker concentrations tested. Instead, a Tom70-adduct was observed at approx. 85 kDa in the presence of 500 μ M and 1 mM s-SMCC (Figure 5A, lanes 2 and 3). The cross-link could represent an internal contact as observed with purified YS585AA₁₁₀, or some other interaction on the membrane, but in any case it is too small to be homodimeric Tom70. This first report of cross-linking endogenous human Tom70 shows that human Tom70 exists predominantly as a monomer on the outer mitochondrial membrane. Human Tom70 is therefore distinct from its yeast homologue, which cross-links as dimers on the membrane [19,21].

We also compared endogenous human Tom70 on BN-PAGE, which had been applied to its yeast counterpart [19]. We solubilized the HeLa cell mitochondria in 1% digitonin, separated the lysate on BN-PAGE and then detected Tom70 by immunoblotting. Human Tom70 migrated in a wide band slightly above 140 kDa on BN-PAGE, larger than expected for monomers (Figure 5B), but similar to the migration of the yeast homologue. However, addition of the cross-linker s-SMCC had no effect on the migration of human Tom70 on BN-PAGE (results not shown), and such treatment had been shown not to produce a homodimeric cross-link (Figure 5A). This led us to believe that monomeric full-length Tom70 may migrate abnormally slowly on BN-PAGE.

To address this using a different approach, we compared the WT Tom70 in its native environment with the YS585AA mutant form that favoured the monomeric state. Full-length WT and YS585AA Tom70 were radiolabelled by cell-free translations (Figure 5C, lanes 1 and 4). After integration into isolated HeLa cell mitochondria, the mitochondria containing Tom70 WT or YS585AA were treated with 1% DMSO or 1 mM s-SMCC and analysed by electrophoresis and autoradiography (Figure 5C, lanes 2, 3, 5 and 6). Again, a lower-molecular-mass adduct was present at 85 kDa on SDS/PAGE, but no homodimeric cross-link was observed for radiolabelled human Tom70 WT and YS585AA. In parallel experiments, radiolabelled WT and YS585AA Tom70 integrated into HeLa cell mitochondria were analysed by BN-PAGE (Figure 5D), and both migrated identically with endogenous Tom70 (Figure 5B). These results also suggested a slow abnormal migration of monomeric Tom70.

Furthermore, we investigated the behaviour of our purified recombinant Tom70 WT₁₁₀ and YS585AA₁₁₀ on BN-PAGE. We had already shown that WT₁₁₀ exists in equilibrium between monomer and dimer, whereas YS585AA₁₁₀ is mostly monomeric. Nonetheless, when subjected to BN-PAGE under the same conditions used for endogenous HeLa cell Tom70, both of them migrated slightly below 140 kDa (Figure 5E), and the addition of the cross-linker SMCC had no apparent effect on such migration (results not shown). This result was in reasonable agreement with the full-length forms (Figures 5B and 5D), given that the recombinant proteins have smaller molecular masses. This was also intriguing because a difference in migrations on BN-PAGE between monomeric and dimeric Tom70 populations was not detected. To investigate this more closely, we treated WT₁₁₀ and YS585AA₁₁₀ with SMCC (Figure 2C, lanes 3 and 4,) and resolved them on BN-PAGE in the first dimension followed by SDS/PAGE in the second dimension (Figure 5F). In addition to the main band in the centre, WT₁₁₀ formed a homodimeric cross-link at approx. 150 kDa on SDS/PAGE (Figure 5F, XL₂, left panel). Although this homodimeric cross-link was well separated in the second dimension, it migrated only slightly slower than the non-cross-linked

Tom70 on the BN-PAGE first dimension. In agreement with this, YS585AA₁₁₀ mainly resolved as a spot in the centre similar to that of WT (Figure 5F, right panel), with only a trace amount of homodimeric cross-link visible on overexposure of the immunoblot. We conclude that the monomeric and dimeric forms of Tom70 had similar and overlapping migrations in BN-PAGE. This may be due to the asymmetric shape of the monomer, or the flexibility of different monomer conformations [18,25].

Taken together, our results suggest that cross-linking rather than BN-PAGE migration is the more reliable test of Tom70 homodimerization. By this measure, we showed that the conditions on the outer mitochondrial membrane are normally unfavourable for dimer formation, perhaps because the concentration of human Tom70 on the membrane is too low. Our co-precipitation and cross-linking experiments of stable preprotein–Tom70 complexes also suggest that the monomer is the functional state capable of binding both chaperones and preproteins. However, Tom70 oligomerization under transiently high local concentrations at subsequent import steps is still possible.

DISCUSSION

The present study provides the first experimental demonstration that the biological function of Tom70 is affected by its oligomeric state. Previous biophysical studies of recombinant yeast Tom70 provided convincing evidence that it was solely monomeric, and functional for binding peptides from chaperone and preprotein. Yet, because dimers of yeast Tom70 could only be observed by cross-linking of endogenous protein on membranes, the relative activity of the putative dimers could not be directly tested [18,19,23]. In the present study, we find that dimers of recombinant human Tom70 can in fact be detected, but that human Tom70 monomers are more active than dimers in interacting with preproteins. This is consistent with the predominantly monomeric form of Tom70 observed on human mitochondria. Overall, our results support the idea that Tom70 is functionally monomeric [18], with higher-order oligomers formed only transiently or under particular conditions.

Human Tom70 diverges from its yeast homologue in many regions of primary sequence, and it is not surprising that aspects of its biochemical mechanism also differ. Functional surfaces including the TPR clamp domain are moderately, not absolutely, conserved between the proteins, and it has been shown that specificity of chaperone binding is different between human and yeast Tom70 [12]. The surface of human Tom70 that is predicted to form the interdomain and/or intersubunit contact is also divergent from the yeast protein, with the critical Tyr⁵⁸⁵–Ser⁵⁸⁶ residues being identical only in metazoan Tom70s (the equivalent residues are isoleucine and threonine in *S. cerevisiae* Tom70 and threonine and threonine in Tom71). Differences in oligomer formation between human and yeast Tom70 may also be expected. Indeed, AUC SV and SE experiments (Figures 1B and 1C) and SEC-MALS data (Figure 2B) established that, although soluble human Tom70 monomers were the majority of the population, dimers were unambiguously evident. The double YS585AA mutation in the predicted interdomain interface disrupted homodimerization of recombinant human Tom70, as shown by SEC-MALS and cross-linking (Figures 2B and 2C). This provided us with a means of testing the effect of dimerization on Tom70 function. Preprotein targeting to Tom70 was more efficient with the monomeric form of the receptor, but not by changing

Tom70 interaction with chaperones. Instead, stronger preprotein–Tom70 cross-links were observed, indicating greater contact between the molecules. These results suggest that human Tom70 receives and interacts with preproteins in a monomeric state. Therefore, the monomeric mechanism of Tom70 characterized by Mills et al. [18] is fundamentally conserved from yeast to humans.

In monomeric Tom70, the YS585AA mutation could also promote dissociation of the TPR clamp and C-terminal domains and increase the overall flexibility of the protein. We were unable to directly observe conformational differences within the monomer populations, as the bulk biophysical properties of the conformers were apparently too similar. It can still be speculated that the domain-dissociated monomer is more efficient at contacting preprotein than the domain-docked form, because the increased flexibility may allow the C-terminal domain to adapt its structure to that of the preprotein ligand. Also, we cannot distinguish the domain arrangement within our observed dimers. Although it is possible that the subunits are in the closed conformation first proposed by Wu and Sha [22], it seems more likely that the dimers are made up of subunits in the elongated open form, in the interpretation of Mills et al. [18]. The dimer would then resemble that depicted in Figure 2(A). The latter arrangement would allow dimer formation without major internal re-orientations of the domains for both human and yeast Tom70.

Recent structural studies of yeast Tom70 [18] and Tom71 [25] support our monomeric model of Tom70 function and outline a generally conserved mechanism. Nonetheless, given that human recombinant Tom70 can form homodimers, we cannot rule out the possibility that homodimeric Tom70 may be present on the outer mitochondrial membrane as a minor species. It was suggested that in a resting state, yeast Tom70 could exist in a homodimeric form to prevent non-specific interactions, and that chaperone docking may be involved in opening Tom70 into the monomeric state before preprotein binding [25]. We suggest an updated model in which human Tom70 interacts with chaperones and preproteins in the monomeric active form, and the homodimeric form may be involved in another mechanistic function perhaps unique to metazoan Tom70s. After preprotein binding, up to six active monomeric receptors may be recruited by one molecule of preprotein in order to proceed to the general import pore [19]. This creates a local environment where high concentrations of the receptor may drive transient homodimerization. The closed conformation of the homodimer could then provide a mechanism for the import receptor to release the preprotein for translocation across the outer mitochondrial membrane. It is also possible that dimerization of Tom70 is induced under certain cellular conditions, perhaps by post-translational modifications, as a means of down-regulating mitochondrial import. New approaches to study Tom70 in the process of preprotein translocation will be needed to establish or refute these ideas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Richard Marcellus, William Wittbold (Wyatt Technology) and the members of Jason Young's laboratory for technical assistance.

FUNDING

This work was supported by the Canadian Institutes of Health Research [grant number MOP-68825], and grants from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Pesquisa e Desenvolvimento (CNPq). J.C.Y. holds a Tier II Canada Research Chair in Molecular Chaperones. L.M.G. holds a FAPESP fellowship.

Abbreviations used

AAC	ADP/ATP carrier
AUC	analytical ultracentrifugation
BMH	bismaleimido-hexane
BN-PAGE	blue native PAGE
DTT	dithiothreitol
GIP	general import pore
Hsc70	heat-shock cognate 70
Hsp	heat-shock protein
MALS	multi-angle light scattering
MBS	<i>m</i> -maleimidobenzoyl- <i>N</i> -hydroxysulfosuccinimide ester
OGC	oxoglutarate carrier
PiC	phosphate carrier
SAXS	small-angle X-ray scattering
SEC	size-exclusion chromatography
SMCC	succinimidyl-4-(<i>N</i> -maleimidomethyl) cyclohexane-1-carboxylate
s-SMCC	sulfo-SMCC
SE	sedimentation equilibrium
SV	sedimentation velocity
Tim	translocase of the mitochondrial inner membrane
Tom	translocase of the mitochondrial outer membrane
TPR	tetratricopeptide repeat
WT	wild-type

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Figure 1. Biophysical analysis of Tom70₁₁₀

(A) To obtain the CD spectrum of Tom70₁₁₀, the residual molar ellipticity (Θ) was measured from 260 to 200 nm at 20 °C. Deconvolution indicated a percentage of $66 \pm 4\%$ of α -helix. (B) $c(s)$ distribution from SV analyses performed with Tom70₁₁₀ at 500 $\mu\text{g/ml}$ (broken line) and 1500 $\mu\text{g/ml}$ (continuous line). Peaks had sedimentation coefficients of 3.8 S and 6.2 S, consistent with molecular masses of approx. 60 kDa and 120 kDa with $f/f_0 = 1.4$. (C) SE analyses were carried out at 150 $\mu\text{g/ml}$ (left panels) and 1500 $\mu\text{g/ml}$ (right panels) at 10 000 rev./min at 20 °C.

Figure 2. The mutation YS585AA disrupts homodimerization of Tom70 $\Delta 110$

(A) An intertwined dimeric model of Tom70 was generated with the human homology model (ribbon structure) superimposed on to the yeast Tom70 crystal structure (PDB: 2GW1) used as template (stick structure). A double mutation at the dimerization interface, YS585AA (black spheres), was generated in human Tom70. Mutation R192A (dark grey spheres) in the TPR clamp is also indicated in the structure. (B) Recombinant Tom70 WT $\Delta 110$ (upper panel) and YS585AA $\Delta 110$ (lower panel) were analysed by SEC-MALS. The elution profile from a Superdex 200 10/300 column (straight line) is plotted with the corresponding molecular masses (squares). Elution volumes of the peaks are indicated. Double arrows denote areas under the peaks selected for molecular mass estimations. (C) Cross-linking of recombinant WT $\Delta 110$ and YS585AA $\Delta 110$ performed with 1% DMSO control (lanes 1 and 2) or 100 μ M SMCC (lanes 3 and 4). The higher molecular mass cross-link was observed at approx. 150 kDa (arrow XL₂) for WT $\Delta 110$ (lane 3) but not for YS585AA $\Delta 110$ (lane 4). (D) WT $\Delta 110$ was cross-linked with 100 μ M SMCC and then resolved on a Superdex 200 10/300 column. Fractions between 11.7 and 15.3 ml were pooled, trichloroacetic acid precipitated and analysed by SDS/PAGE and Coomassie Blue staining. The homodimeric cross-link (arrow XL₂) eluted in earlier fractions, whereas in later fractions only un-cross-linked Tom70 was present.

Figure 3. Co-precipitation of Tom70₁₁₀ with preproteins and chaperones

(A) OGC was radiolabelled by cell-free translation (lane 1) and then co-precipitated with nickel–Sephacel in the absence (lane 2) or presence of the His-tagged cytosolic domain of human Tom70 WT₁₁₀, YS585AA₁₁₀ or R192A₁₁₀ (lanes 3–5). The final concentration of the His-tagged proteins was 5 μ M, which was within the biological concentration range of Hsp90 and its co-chaperones. Quantification of the OGC band showed OGC bound by YS585AA₁₁₀ was increased to 140% of WT₁₁₀ at 5% level of significance. (B) Similarly, radiolabelled PiC was recovered by YS585AA₁₁₀ and the amount bound was increased to 120% of WT₁₁₀ at 1% level of significance. Co-precipitation of radiolabelled Hsc70 (C) and Hsp90 (D) showed no significant difference in chaperone docking between WT₁₁₀ and YS585AA₁₁₀. Significance level (P) was calculated based on the Student's t test with a minimum of three independent experiments ($n = 3$).

Figure 4. Cross-linking of preprotein-Tom70₁₁₀ complexes

(A) Upper panel: radiolabelled PiC (lane 1) was co-precipitated with His-tagged WT₁₁₀ (lane 2) or YS585AA₁₁₀ (lane 5) and recovered by nickel–Sepharose as described in Figure 3. After incubation with BMH, stable complexes were washed in the absence (lanes 3 and 6) or presence of SDS (lanes 4 and 7). Bands that are SDS-resistant represent direct interactions (XL arrows). A cross-link of particular interest in YS585AA₁₁₀ is marked (XL_a). Lower panel: a magnified view of the cross-linked bands at the higher molecular masses. (B) Radiolabelled OGC was co-precipitated and cross-linked with BMH (XL arrows). A magnified view of the cross-linked bands is shown. Lanes are numbered as in (A).

Figure 5. The native state of human Tom70

(A) Mitochondria from HeLa cells were incubated with 1% DMSO (lane 1) or cross-linked with 1000, 500, 100, 50, 10, 5 or 1 μ M s-SMCC (lanes 2–8) and then analysed by SDS/PAGE and immunoblotting against Tom70. (B) HeLa cell mitochondria were solubilized in 1% digitonin and resolved on 6–16.5% BN-PAGE gels. Endogenous Tom70 was visualized by immunoblotting. (C) Full-length human Tom70 WT or YS585AA were radiolabelled by cell-free translation (lanes 1 and 4). After insertion into HeLa cell mitochondria, reactions were incubated with 1% DMSO (lanes 2 and 5) or 1 mM s-SMCC (lanes 3 and 6) and analysed by SDS/PAGE and autoradiography. (D) Radiolabelled human Tom70 WT or YS585AA was inserted into HeLa cell mitochondria, solubilized in 1% digitonin and analysed on BN-PAGE and autoradiography as described. (E) WT Δ_{110} and YS585AA Δ_{110} were resolved on BN-PAGE in the presence of 1% digitonin and then visualized by Coomassie Blue staining. Native molecular mass markers are indicated. (F) WT Δ_{110} (left panel) and YS585AA Δ_{110} (right panel) were cross-linked with 100 μ M SMCC, resolved on BN-PAGE in the first dimension and SDS/PAGE (8% gels) in the second dimension,

followed by immunoblotting against Tom70. The homodimeric cross-link (XL₂) was well separated on SDS-PAGE but not on BN-PAGE.