Evidence of Distinct Channel Conformations and Substrate Binding Affinities for the Mitochondrial Outer Membrane Protein Translocase Pore Tom40*

Background: Nearly all nascent mitochondrial proteins are transported by the translocase of the outer membrane (TOM) complex.

Results: The core Tom40 β-barrel domain exhibits four conductive levels and three distinct substrate binding affinities.

Conclusion: Tom40 interactions with presequence substrates depend upon the channel’s conformation.

Significance: Conformational rearrangements in Tom40 may regulate substrate interactions.

Mitochondria originated from an endosymbiosis event between ancient prokaryotic cells (1, 2). Although a minimal amount of genetic material was retained as mitochondrial DNA, the vast majority of mitochondrial proteins are now encoded in the nuclear genome. Thus, most mitochondrial proteins must be transported across the outer mitochondrial membrane for sorting to their correct sub-mitochondrial location (3–6). The translocation and membrane insertion of unfolded mitochondrial proteins are accomplished by several multiprotein complexes in the outer and inner membranes. These complexes recognize mitochondrial targeting information encoded in presequences found on mitochondrial precursor protein substrates (5). The translocase of the outer membrane (TOM5 complex) is composed of a central pore formed by the essential protein Tom40, along with the substrate receptor proteins Tom70, Tom22, and Tom20, and the small accessory proteins Tom5, Tom6, and Tom7. Elegant studies have revealed the general organization of the TOM complex and have monitored the progressive stages in the biogenesis of mitochondrial proteins (7–13). However, molecular detail of the Tom40 translocation mechanism remains to be understood (14–16).

Tom40 has to provide transitory binding sites for its substrates, which generally consist of basic and amphipathic presequence matrix proteins. It also must stabilize unfolded proteins as they transit through its translocation pore. A structural model where two Tom40 pores act in concert as a dimeric protein complex best accounts for the observed biochemical and electrophysiological data. These results provide the first evidence for structurally distinct Tom40 conformations playing a role in substrate recognition and therefore in transport function.

Nearly all mitochondrial proteins are coded by the nuclear genome and must be transported into mitochondria by the translocase of the outer membrane complex. Tom40 is the central subunit of the translocase complex and forms a pore in the mitochondrial outer membrane. To date, the mechanism it utilizes for protein transport remains unclear. Tom40 is predicted to comprise a membrane-spanning β-barrel domain with conserved α-helical domains at both the N and C termini. To investigate Tom40 function, including the role of the N- and C-terminal domains, recombinant forms of the Tom40 protein from the yeast Candida glabrata, and truncated constructs lacking the N- and/or C-terminal domains, were functionally characterized in planar lipid membranes. Our results demonstrate that each of these Tom40 constructs exhibits at least four distinct conductive levels and that full-length and truncated Tom40 constructs specifically interact with a presequence peptide in a concentration- and voltage-dependent manner. Therefore, neither the first 51 amino acids of the N terminus nor the last 13 amino acids of the C terminus are required for Tom40 channel formation or for the interaction with a presequence peptide. Unexpectedly, substrate binding affinity was dependent upon the Tom40 state corresponding to a particular conductive level. A
homology model of Tom40 based on a distantly related protein, the voltage-dependent anion channel, has thus far only provided limited insights into Tom40's molecular function (17–19). Three-dimensional models of Tom40 based on mouse VDAC1 (20–23) predict a structure composed of 19 transmembrane-β-strands forming a β-barrel. In these models, the second of two structurally conserved N-terminal α-helices is placed within the lumen of the Tom40 β-barrel, as observed for VDAC1’s single N-terminal α-helix, and is suggested to stabilize the barrel wall (20). The structural models also suggest that the Tom40 protein from the yeast Saccharomyces cerevisiae and the filamentous fungus Neurospora crassa will be similar to that found in humans and other mammals (20, 22, 24).

In this work, we have investigated the impact that the N and C termini of Tom40 have on its function. A full-length Tom40 protein from the yeast Candida glabrata and engineered truncation mutants lacking either terminus were expressed as inclusion bodies in Escherichia coli, refolded, purified, and reconstituted into planar lipid membranes. In this membrane environment, the channel properties and substrate binding of the Tom40 protein constructs were analyzed under various conditions.

Our results demonstrate that all the investigated Tom40 constructs form channels with four well defined conductive levels. All Tom40 constructs were shown to be functional, even in the absence of accessory Tom proteins, by interacting with a specific presequence peptide in a concentration-dependent manner. Surprisingly, we found that substrate binding affinity appears to be regulated by the Tom40 channel state corresponding to a particular conductive level. These studies therefore reveal that the majority of the Tom40 N-terminal domain, including an evolutionarily conserved α-helix, is not required for presequence interaction with Tom40. Furthermore, these results suggest a model in which Tom40 functions as a dimer and that substrate binding is governed by the distinct conformations that this dimer adopts.

Experimental Procedures

Sequence Alignments and Secondary Structure Predictions—The amino acid sequences of Tom40 proteins from S. cerevisiae (NCBI accession number DAA10102), N. crassa (NCBI accession number EAA32309) and C. glabrata (NCBI accession number CAB60054) were analyzed for sequence conservation and structural similarity. Linear sequence alignments and conservation comparisons were generated utilizing PRALINE (25) and Clustal W (26) servers. Secondary structure predictions were made using a combination of the PRALINE and PSIPRED (27) servers.

C. glabrata Tom40 Expression—The amino acid sequence of the C. glabrata Tom40 protein (Tom40wt-FL), as well as a mutant in which all four cysteine residues were converted to alanine (C160A, C321A, C336A, and C350A, Tom40ca-FL), were codon-optimized for bacterial expression and synthetically created in a pUC57 plasmid (nucleotide sequences available upon request) (Genscript, Piscataway, NJ).

The C. glabrata Tom40-coding sequence was inserted into an isopropyl 1-thio-β-D-galactopyranoside-inducible pET28 vector (Novagen/EMD Millipore, Billerica, MA), with and without an N-terminal His₆ purification tags, using ligation-independent cloning methods (28). After sequence validation of the Tom40ca-FL construct plasmid, the same ligation-independent cloning methodology was employed to create N- and C-terminal truncation constructs (primer sequences available upon request). The amino acid sequences of the C. glabrata Tom40 constructs (cysteine residues are underlined) were as follows.

Tom40wt-FL, MSAPEVKSTAIAPPISLNEQDSKSSWSANPLSSFVQDRTYRLHARQLSLVLNPVGTENLKEVRDVFLSQYFTGGLRARLDNKFLTNPAFTSHTSFGTSLPNYAFASLFANDLNFMQGIDNSFLSRLGMNYWNKNNISKLNLQIANGQPTMCQQLEQDYAQASDSSNLKFNSISNNSTLTGVYYGLQLSISQPLAVGEVLYSRAKTAQTPDSGSVYLTRVSPKQDWSQGLQLQANGALVASFWRVKAPVNEAGIETTLQAGMPIPTDPVMPGTPQIPQETEISTTIGAKYERYQSVRYQVSYSVDSNGKIGFRLKLPLTLSVLFAGEIDQFKQESKIGAGLQFETAGNQQELLMMMQQGLDAGNPLQALPEM.

Tom40ca-FL, MSAPEVKSTAIAPPISLNEQDSKSSWSANPLSSFVQDRTYRLHARQLSLVLNPVGTENLKEVRDVFSLSQYFTGGLRARLDNKFLTNPAFTSHTSFGTSLPNYAFASLFANDLNFMQGIDNSFLSRLGMNYWNKNNISKLNLQIANGQPTMAQLEQDYAQASDSSNLKFNSISNNSTLTGVYYGLQLSISQPLAVGEVLYSRAKTAQTPDSGSVYLTRVSPKQDWSQGLQLQANGALVASFWRVKAPVNEAGIETTLQAGMPIPTDPVMPGTPQIPQETEISTTIGAKYERYQSVRYQVSYSVDSNGKIGFRLKLPLTLSVLFAGEIDQFKQESKIGAGLQFETAGNQQELLMMMQQGLDAGNPLQALPEM.

Tom40ca-319, MSAPEVSKITAIPISLNEQDSKSSWSANPLSSFVQDRTYRLHARQLSLVLNPVGTENLKEVRDVFLSQYFTGGLRARLDNKFLTNPAFTSHTSFGTSLPNYAFASLFANDLNFMQGIDNSFLSRLGMNYWNKNNISKLNLQIANGQPTMAQLEQDYAQASDSSNLKFNSISNNSTLTGVYYGLQLSISQPLAVGEVLYSRAKTAQTPDSGSVYLTRVSPKQDWSQGLQLQANGALVASFWRVKAPVNEAGIETTLQAGMPIPTDPVMPGTPQIPQETEISTTIGAKYERYQSVRYQVSYSVDSNGKIGFRLKLPLTLSVLFAGEIDQFKQESKIGAGLQFETAGNQQELLMMMQQGLDAGNPLQALPEM.

Tom40ca-332, MSAPEVSKITAIPISLNEQDSKSSWSANPLSSFVQDRTYRLHARQLSLVLNPVGTENLKEVRDVFLSQYFTGGLRARLDNKFLTNPAFTSHTSFGTSLPNYAFASLFANDLNFMQGIDNSFLSRLGMNYWNKNNISKLNLQIANGQPTMAQLEQDYAQASDSSNLKFNSISNNSTLTGVYYGLQLSISQPLAVGEVLYSRAKTAQTPDSGSVYLTRVSPKQDWSQGLQLQANGALVASFWRVKAPVNEAGIETTLQAGMPIPTDPVMPGTPQIPQETEISTTIGAKYERYQSVRYQVSYSVDSNGKIGFRLKLPLTLSVLFAGEIDQFKQESKIGAGLQFETAGNQQELLMMMQQGLDAGNPLQALPEM.

Tom40ca-369, MSAPEVSKITAIPISLNEQDSKSSWSANPLSSFVQDRTYRLHARQLSLVLNPVGTENLKEVRDVFLSQYFTGGLRARLDNKFLTNPAFTSHTSFGTSLPNYAFASLFANDLNFMQGIDNSFLSRLGMNYWNKNNISKLNLQIANGQPTMAQLEQDYAQASDSSNLKFNSISNNSTLTGVYYGLQLSISQPLAVGEVLYSRAKTAQTPDSGSVYLTRVSPKQDWSQGLQLQANGALVASFWRVKAPVNEAGIETTLQAGMPIPTDPVMPGTPQIPQETEISTTIGAKYERYQSVRYQVSYSVDSNGKIGFRLKLPLTLSVLFAGEIDQFKQESKIGAGLQFETAGNQQELLMMMQQGLDAGNPLQALPEM.

Tom40ca-332, MLVPVGTENLKEVRDVFLSQYFTGGLRARLDNKFLTNPAFTSHTSFGTSLPNYAFASLFANDLNFMQGIDNSFLSRLGMNYWNKNNISKLNLQIANGQPTMAQLEQDYAQASDSSNLKFNSISNNSTLTGVYYGLQLSISQPLAVGEVLYSRAKTAQTPDSGSVYLTRVSPKQDWSQGLQLQANGALVASFWRVKAPVNEAGIETTLQAGMPIPTDPVMPGTPQIPQETEISTTIGAKYERYQSVRYQVSYSVDSNGKIGFRLKLPLTLSVLFAGEIDQFKQESKIGAGLQFETAGNQQELLMMMQQGLDAGNPLQALPEM.

Tom40ca-319, MLVPVGTENLKEVRDVFLSQYFTGGLRARLDNKFLTNPAFTSHTSFGTSLPNYAFASLFANDLNFMQGIDNSFLSRLGMNYWNKNNISKLNLQIANGQPTMAQLEQDYAQASDSSNLKFNSISNNSTLTGVYYGLQLSISQPLAVGEVLYSRAKTAQTPDSGSVYLTRVSPKQDWSQGLQLQANGALVASFWRVKAPVNEAGIETTLQAGMPIPTDPVMPGTPQIPQETEISTTIGAKYERYQSVRYQVSYSVDSNGKIGFRLKLPLTLSVLFAGEIDQFKQESKIGAGLQFETAGNQQELLMMMQQGLDAGNPLQALPEM.

T7 Express competent E. coli cells (New England Biolabs, Ipswich, MA) were transformed and grown in the presence of...
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50 μg/ml kanamycin at 37 °C to an A560 of 0.6–0.8, induced with the addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and they were incubated for 3–4 h at 37 °C.

Isolation of Mitochondria and C. glabrata Tom40 Import and Assembly—Mitochondria were isolated from S. cerevisiae as described previously (29). At least three preparations of each C. glabrata construct were tested for protein import and assembly rates.

The methods for protein translation, import assays, and protease shaving for topological assessment have been previously described (30–32). Plasmid (pSP65) constructs were created using the same nucleotide sequences as above for the full-length C. glabrata wild-type and Cys → Ala mutant sequences. These constructs were transcribed in vitro using SP6 RNA polymerase (Promega, Madison, WI). RNA was translated in vitro using nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of 35S-labeled methionine (MP Biomedicals, Santa Ana, CA). Translated 35S-labeled protein was incubated with isolated mitochondria for time points up to 90 min at 25 °C in import buffer (0.6 M sorbitol, 50 mM KCl, 25 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, 5 mM methionine). After one wash in import buffer, mitochondria were either subjected to SDS-PAGE or native gel electophoresis and solubilized in 1.0% digitonin in lysis buffer (20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1% phenylmethylsulfonyl fluoride). After 15 min on ice with intermittent vortexing, the insoluble components were collected by centrifugation, and the soluble supernatant was combined with BN-PAGE sample buffer (5% Coomassie Blue G, 500 mM aminocaproic acid in 100 mM bis-tris (pH 7.0)) and subjected to BN-PAGE using a 2.5–3.0 mg/ml. Denatured Tom40 IBs were diluted 10-fold dialysis against 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SB3-12, and 0.01% NaN3, and 0.5 mM TCEP. Blue dextran, ovalbumin, bovine serum albumin, and chymotrypsinogen A standard proteins (GE Healthcare) were used to calibrate the size exclusion column.

Circular Dichroism—The SB3-12 detergent refolding of recombinant Tom40 was monitored by circular dichroism analyses. Peak gel filtration fractions (−0.6–1.0 mg/ml pure protein in 0.2% SB3-12) were diluted to 0.25 mg/ml in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SB3-12, and 0.01% NaN3, CD spectra of the samples were recorded on a Jasco J-810 spectropolarimeter using a 0.1-cm path length cell at 20 °C. After subtracting a scan of buffer alone, CD traces were processed using the Spectra Manager 2 software provided with the instrument. Secondary structure content was determined using the CDNN program (33). All subsequent experimentation was performed on Tom40 reconstituted into lipid membranes, which may further affect secondary structure. Therefore, a more definitive validation of Tom40 construct refolding proficiency was demonstrated by the measurements of functional substrate binding.

Proteoliposome Preparation—1-α-Phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) was solubilized in chloroform at 10 mg/ml and lyophilized under N2 gas. The lipid was then resuspended at 20 mg/ml in 20 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA with vortexing and bath sonication. The lipid mixture was solubilized with the addition of SB3-12 detergent, at a final concentration of 2.0%. Peak gel filtration fractions of refolded Tom40 were then added at a final weight ratio of 100:1, lipid/protein. The lipid/Tom40 mixture was incubated on ice for 30 min, and detergent was removed by three rounds of exposure to BioBeads SM-2 (Bio-Rad), at a weight ratio of 1:30 lipid/BioBeads. BioBeads were subsequently removed with low speed centrifugation, and the supernatant containing Tom40 proteoliposomes was then centrifuged at 109,000 × g. The isolated Tom40 proteoliposomes were gently resuspended in 20 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, at 1/5 the volume of the initial reconstitution. Tom40 proteoliposomes were rapidly frozen in liquid N2 and stored at −80 °C until use in electrophysiology measurements.

Channel Reconstitution—Planar bilayer membranes were formed from soybean polar lipid extract (Avanti Polar Lipids, Alabaster, AL) dissolved in pentane, using the lipid monolayer opposition technique, on a 70–80-μm diameter orifice in the 15-μm-thick Teflon partition that separated two (cis and trans) compartments of the experimental cell, as described previously (34). Potential is defined as positive when it is greater at the side. Tom40 insertion was achieved by adding ~0.1 μl of the freshly thawed proteoliposome sample to the cis side solution. A voltage of −100 to −150 mV was applied until a stepwise increase of stable current indicated insertion of Tom40. To achieve effective insertion, all experiments were
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performed in asymmetrical aqueous buffer conditions (to achieve effective insertion of Tom40), with 250 mM KCl, 5 mM CaCl₂ in the cis compartment and 20 mM KCl in trans, buffered with 5 mM HEPES (pH 7.4) on both sides. All measurements were carried out at room temperature (21 ± 1.5 °C). Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in a voltage clamp mode as described (34). A pair of Ag/AgCl electrodes immersed in 2M KCl, 1.5% agarose bridges was used for applying voltages to membranes and for recording transmembrane currents. The amplifier output signal was filtered by an in-line low pass eight-pole Butterworth filter (model 9002; Frequency Devices, Haverhill, MA) at 15 kHz and applied to all current records. Open probability was calculated using Clampfit 10.2. Ion selectivity was inferred from the potential corresponding to the intersection of the current-voltage curve with the zero-current level for each individual conductive level. This “reversal potential” was used to calculate permeability ratios between K⁺/Cl⁻, P_K/P_Cl, as described previously (35).

Peptide Synthesis—Model mitochondrial precursor peptides, including the first 31 amino acids of the β-subunit of the S. cerevisiae F₁-ATP synthase (pF1, Ac-MVLPRLYTATSRAAF-KAAKQSAPLLSTSWKR-NH₂) and an artificial nonspecific negative control peptide with an α-helical structure and positive charge (SynB2, MLSRQQSQRQSRQQSQRQSYLL), were synthesized by the Facility for Biotechnology Resources at the

FIGURE 1. Fungal Tom40 alignments and secondary structure predictions. Tom40 sequences from S. cerevisiae, N. crassa, and C. glabrata were analyzed for sequence conservation and secondary structure prediction using a combination of the PRALINE alignment toolbox (25), ClustalW (26), and the PSIPRED protein structure prediction server (27). Consensus symbols below the alignment indicate either a fully conserved residue (*), a conservation of strongly similar residues (:), or a conservation of weakly similar residues (.). A consensus between the PRALINE and PSIPRED secondary structures is illustrated schematically as red cylinders for α-helical segments and blue arrows for β-strand segments. Cysteine residues are highlighted in yellow.
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Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD. Peptide purity was verified by HPLC prior to lyophilization. Peptides were reconstituted in H2O at a concentration of 2 mM and frozen at −20 °C until used in electrophysiology experiments.

Results

Evolutionarily Conserved Structural Features of Tom40—Secondary structural analyses for Tom40 sequences from the fungal species C. glabrata, S. cerevisiae, and N. crassa are consistent with previous suggestions that Tom40 proteins comprise a membrane-inserted, 19-stranded β-barrel domain with two α-helical domains at the N-terminus and one at the C-terminus (Fig. 1). The two N-terminal α-helical segments are predicted to be a conserved feature in protozoan, yeast, insect, primate, and plant Tom40 sequences. However, homology models are only able to predict an inter-lumen location for the second N-terminal α-helix, because VDAC1 lacks a homologous sequence for the first predicted α-helix (20). Expression and refolding studies on Tom40 protein sequences from seven species of fungi and eight additional species representing a diverse set of eukaryotes revealed that Tom40 from C. glabrata yielded the highest amount of detergent-soluble protein (data not shown). This yeast is a close relative of S. cerevisiae (36), and the Tom40 proteins from the two species are ~80% identical and ~88% similar at the amino acid sequence level. C. glabrata Tom40 is more distantly related to the N. crassa Tom40 (~36% identical and ~55% similar) (Fig. 1). Several cysteine residues are found in each Tom40 sequence, including one (corresponding to C. glabrata C321) that is highly conserved in these species, and even in evolutionary divergent species such as Giardia (data not shown). The importance of these cysteine residues is not known, but their presence was potentially problematic for the heterologous expression and purification of recombinant Tom40 constructs.

C. glabrata Tom40 Import and Assembly—A Cys → Ala replacement mutant (C160A, C321A, C336A, and C350A) was constructed and compared with the wild-type (WT) C. glabrata Tom40 for its ability to form the core of a mature TOM complex in the mitochondrial outer membrane. 35S-Radiolabeled versions of the Tom40 protein from C. glabrata were synthesized in vitro transcription and translation and subjected to import, assembly, and topological assays in mitochondria isolated from S. cerevisiae (Fig. 2). As demonstrated in BN-PAGE assays, both the WT and Cys → Ala mutant C. glabrata Tom40 proteins were imported and assembled into the mature TOM complex with a similar time course and efficiency as observed for Tom40 from S. cerevisiae (Fig. 2A). A difference was noted only with regard to the stability of assembly intermediate II. This ~100-kDa complex contains Tom40, Tom5, and Tom6, at a stage prior to their maturation into the final TOM complex (37, 38), and these results suggest that cysteine residues in Tom40 may be important in the stabilization of this assembly intermediate. Both the WT and Cys → Ala mutant forms of Tom40 from C. glabrata were resistant to proteinase K treatment unless the outer membrane was ruptured by swelling, in the same fashion as Tom40 from S. cerevisiae (Fig. 2B). Extraction using sodium carbonate verified that the C. glabrata WT

![Image](https://via.placeholder.com/150)

FIGURE 2. A cysteine-less Tom40 is assembled into a mature TOM complex. A, mitochondria from S. cerevisiae (100 μg of protein per lane) were incubated with 35S-labeled ScTom40, CgTom40wt-FL, or CgTom40ca-FL at 25 °C for the indicated times (minutes), and then samples were analyzed by BN-PAGE followed by phosphorimage analysis. The assembly intermediates and the mature TOM complex are labeled. B, after import of the indicated 35S-labeled proteins, mitochondria (100 μg of protein per lane) were isolated and treated with proteinase K (PK +) in a hypotonic buffer to swell and rupture the organelles into “mitoplasts” (SW +) or in an isosmotic buffer to retain the integrity of the outer membrane (SW −). After addition of PMSF to inhibit the proteinase, the samples were analyzed by SDS-PAGE and phosphorimage analysis (upper panel) or SDS-PAGE and immunoblotting (lower panel). Controls for proteinase function and mitochondrial integrity are included; Tom70 is an outer membrane control and is accessible to proteinase in intact mitochondria; Mdj1p is a matrix-located protein protected by the inner membrane. Immunoblot analysis of the endogenous ScTom40 is also shown. C, after import of the indicated 35S-labeled proteins, mitochondria (100 μg of protein per lane) were treated with proteinase K and then isolated and incubated with 0.1 mM Na2CO3 (pH 11.5), and the extracted membranes were re-isolated by ultracentrifugation. Duplicate samples of the total material (T), the extract supernatant (S) and the extracted membranes (P) were analyzed by SDS-PAGE and phosphorimage analysis (upper panel) or SDS-PAGE and immunoblotting (lower panel).

...and Cys → Ala mutant Tom40s behave as integral membrane proteins (Fig. 2C). Taken together, these findings provide evidence that the Cys → Ala mutant Tom40 is structurally equivalent to the wild-type Tom40 as the core subunit of a native TOM complex.

C. glabrata Tom40 Expression, Refolding, and Purification—Several constructs for full-length and truncation mutants of C. glabrata Tom40 were generated as follows: full-length Tom40

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Tom40 construct design and purification. The Tom40 protein from C. glabrata is 382 amino acids in length, with a calculated molecular mass of ~41.7 kDa. A, schematic representation of the cysteine-less full-length Tom40 (Tom40ca-FL) and truncation mutant constructs (Tom40ca-369, Tom40ca-332, and Tom40ca-319) used in this study. Secondary structure predictions are indicated as in Fig. 1. Beginning and ending amino acids of the predicted domains are indicated below. B, representative SDS-PAGE resolution and Coomassie staining of detergent-soluble and purified Tom40 constructs. Although the recombinant Tom40 constructs were initially designed with N-terminal 6× histidine tags (H6-Tom40wt-FL and H6-Tom40ca-FL), this purification approach proved unnecessary, and subsequent constructs had no affinity tags. C, typical size exclusion profiles (in 0.2% SB3-12 detergent) of the non-tagged Tom40 wild-type and mutant constructs. CD spectra (D) and SDS-PAGE (E) of non-tagged Tom40ca-FL and Tom40ca-319 protein preparations are shown. Samples were subjected to SDS-PAGE on 29% homogeneous PhastGel and visualized by staining with PhastGel® Blue R (Sigma). M denotes molecular mass standards in kDa.
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gent-stabilized Tom40wt-FL resolved on size exclusion chromatography at an elution volume equivalent to well characterized ~80-kDa β-barrel membrane proteins solubilized in detergent micelles (39), and sample fractions contained an apparent SDS-resistant dimer (Fig. 3B). This recombinant Tom40 dimer is stabilized by cysteine residues, as the Cys → Ala mutant Tom40ca-FL showed little if any SDS-resistant dimer (Fig. 3B). However, there was not a significant shift in the size exclusion chromatography profile of Tom40ca-FL, suggesting that both the wild-type and Cys → Ala mutant Tom40 proteins purify as dimers in the absence of a denaturant. Multiplex light scattering and glutaraldehyde cross-linking analyses supported the conclusion that each refolded Tom40 construct purified as an ~70–80-kDa dimer, although precise molecular weight determinations were not obtained from light scattering due to the confounding influence of the SB3-12 detergent (data not shown). This behavior of the recombinant Tom40 constructs is thus consistent with a substantial population of the Tom40 pores seen as dimers in electron microscopy imaging of native Tom40 extracted from mitochondrial outer membranes (40–46).

Tom40 constructs were initially designed with an N-terminal hexa-histidine tag to aid purification. However, as the inclusion body starting material was highly enriched for Tom40, immobilized metal affinity chromatography (nickel-nitrolotriacetic acid resin) was minimally beneficial for purity, and utilization of anion exchange chromatography proved more effective. Therefore, to simplify the interpretation of subsequent experiments, the Tom40 constructs were redesigned without histidine tags, and these untagged proteins were used for further experiments. The untagged constructs behaved in the same manner as His-tagged Tom40 in refolding and purification (Fig. 3, B and C).

To qualitatively assess refolding of the recombinant proteins, representative samples of the purified, detergent-soluble full-length and 319-truncated Tom40 constructs were measured for their α-helix and β-sheet content with circular dichroism (CD). CD spectra showed the characteristic α-helical signature with a double minimum at 208 and 222 nm. The refolded Tom40ca-FL was calculated to contain a double minimum at 208 and 222 nm. The refolded CD spectra showed the characteristic spectra and secondary structure estimates (~29% α-helix and ~37% β-sheet (Fig. 3D), values that are consistent with previous reports of N. crassa Tom40 solubilized from the endogenous TOM complex (42). Notably, the Tom40ca-319 truncation mutant produced nearly equivalent circular dichroic spectra and secondary structure estimates (~28% α-helix and ~36% β-sheet), suggesting that elimination of amino acids 1–51 and 370–382 does not disrupt Tom40 refolding.

Reconstituted Tom40 Exhibits Four Conductive Levels—To functionally characterize Tom40, refolded and purified proteins were reconstituted into 1-α-phosphatidylcholine liposomes and then incorporated into planar lipid bilayers separating 250 mM KCl, 5 mM CaCl2 (cis), and 20 mM (trans) KCl solutions. Proteoliposomes were added to the cis compartment to achieve a high yield of Tom40 reconstitution (47). Representative current versus time records for Tom40ca-FL and Tom40ca-319 are shown in Fig. 4, A and B, respectively. A total of four distinct conductive levels were observed for reconstituted Tom40ca-FL, ranging from 123 ± 20 pS (level 1, L1) to 518 ± 34 pS (level 4, L4), in apparent increments of 126 ± 2.5 pS (Table 1). Time-resolved transitions were observed between individual conductive levels (e.g. L1 to L3, L3 to L4, and L2 to L4). One-step transitions were also observed between each level and zero current (Fig. 4, time expansions). Tom40ca-FL channels spontaneously shifted between levels during a single experiment and often remained at one of the levels, most often L1 or L2, for prolonged periods. Each conductive level exhibited characteristic fast-frequency downward current flickering (Fig. 4, time expansions). Importantly, the current-voltage relationship, reversal potential, and ion selectivity characteristics of the Cys → Ala Tom40ca-FL are nearly indistinguishable from those of the wild-type Tom40 (Tom40wt-FL) (Table 1).

The truncated Tom40 constructs (Tom40ca-369, Tom40ca-332, and Tom40ca-319) exhibited nearly identical properties to Tom40ca-FL, displaying four distinct conductive levels (Fig. 5). Additionally, the four levels were separated by increments of ~120 pS (Table 1), and all Tom40 constructs showed characteristic time-resolved transitions between each level and zero current (Fig. 5). Channel recordings routinely displayed a momentary closure and re-opening event, which could be seen best in the L4 recordings. The high frequency flickering of the conductive levels is an inherent characteristic of Tom40 chan-
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TABLE 1
Conductance and ion selectivity of four conductive levels for reconstituted C. glabrata Tom40 constructs
Experiments of Tom40 channel reconstitutions were measured in a 250/20 mM gradient of KCl under a range of applied voltages. Current/voltage (I/V) relationship curves were determined for all observed conductive levels. Reversal potentials and conductance values are the mean of data from the number of independent experiments (shown in parentheses) ± S.E.

| Conductive level (no. of experiments) | Conductance | Reversal potential | Permeability ratio (PK/PCl) |
|--------------------------------------|-------------|-------------------|----------------------------|
| Tom40wt-FL                           | 1 (3)       | 120 ± 27          | -50.2 ± 3.3                | 12.7 ± 5.0                |
|                                      | 2 (4)       | 232 ± 13          | -46.3 ± 1.6                | 8.0 ± 1.0                 |
|                                      | 3 (2)       | 364 ± 4           | -42.8 ± 2.6                | 6.1 ± 1.1                 |
|                                      | 4 (3)       | 486 ± 56          | -44.8 ± 0.9                | 6.7 ± 0.1                 |
| Tom40ca-FL                           | 1 (4)       | 123 ± 20          | -52.8 ± 2.8                | 23.7 ± 11.8               |
|                                      | 2 (7)       | 235 ± 25          | -49.3 ± 5.3                | 11.6 ± 5.8                |
|                                      | 3 (2)       | 371 ± 7           | -47.7 ± 2.1                | 9.3 ± 2.5                 |
|                                      | 4 (2)       | 518 ± 34          | -45.7 ± 1.7                | 7.8 ± 1.2                 |
| Tom40ca-369                          | 1 (3)       | 121 ± 34          | -42.3 ± 2.8                | 8.2 ± 2.9                 |
|                                      | 2 (2)       | 238 ± 8           | -48.6 ± 1.5                | 10.1 ± 1.8                |
|                                      | 3 (6)       | 341 ± 17          | -47.4 ± 3.2                | 9.0 ± 1.9                 |
|                                      | 4 (3)       | 492 ± 95          | -46.6 ± 2.4                | 8.3 ± 2.1                 |
| Tom40ca-332                          | 1 (2)       | 147 ± 23          | -41.7 ± 8.5                | 6.6 ± 3.7                 |
|                                      | 2 (4)       | 278 ± 19          | -48.7 ± 5.5                | 13.1 ± 8.2                |
|                                      | 3 (2)       | 352 ± 10          | -46.5 ± 1.1                | 8.3 ± 0.8                 |
|                                      | 4 (4)       | 479 ± 49          | -43.1 ± 1.7                | 6.4 ± 0.7                 |
| Tom40ca-319                          | 1 (5)       | 120 ± 15          | -42.3 ± 5.3                | 6.6 ± 2.7                 |
|                                      | 2 (5)       | 241 ± 25          | -46.0 ± 6.1                | 7.8 ± 2.7                 |
|                                      | 3 (5)       | 376 ± 38          | -41.8 ± 3.4                | 6.0 ± 1.4                 |
|                                      | 4 (4)       | 486 ± 8           | -45.7 ± 5.5                | 8.7 ± 3.6                 |

FIGURE 5. Tom40ca-FL and the Tom40ca-369, Tom40ca-332, and Tom40ca-319 truncation mutants form channels with four conductive levels of similar conductance and characteristic gating. Representative current traces of four conductive levels fluctuating between that particular level and the closed state were obtained with each Tom40 channel. All experimental conditions are as in Fig. 4. Traces are representative of 3–6 experimental recordings of each conductive level.

Conductance of individual levels was measured as the slope of the current-voltage (I/V) plots. Averaged I/V plots of individual conductive levels for Tom40ca-FL and Tom40ca-319, obtained from two to eight independent experiments for each construct, are shown in Fig. 6. All conductive levels had high cation selectivity, typical for Tom40 (47, 50), with an average permeability ratio (PK/PCl) of 9.4 ± 4.2 (Table 1). The high cation selectivity of Tom40 complements the positively charged presequences of its endogenous substrates such as inner membrane subunit proteins of the electron transport chain enzyme complexes or matrix-bound precursor proteins (51).

Distinct Tom40 Conductive Levels Have Different Substrate Binding Affinities—To investigate the functional properties of these Tom40 constructs, their interaction with a well studied substrate was examined. A peptide consisting of the first 31 amino acids of mitochondrial inner membrane ATPase, F1β (pF1β) was used as a model substrate (40, 42, 48). Addition of pF1β to the cis side of the membrane induced blockage of Tom40 conductance in a concentration-dependent manner, indicating direct interaction of peptide with the Tom40 pore (Fig. 7A) (52). To test the specificity of pF1β binding to Tom40
channels, experiments were performed with SynB2 (53), a synthetic peptide similar in structure and charge to matrix protein precursor sequences but composed of an amino acid sequence not recognized by TOM (47, 54). The addition of up to 30 μM SynB2 did not induce an observable increase in channel closure for any of the Tom40 constructs (representative current records for Tom40ca-369 are shown in Fig. 7B). Furthermore, pF1β retained its ability to induce Tom40 channel closure in the presence of 30 μM SynB2 (Fig. 7B, lower trace). Tom40 channel blockage by pF1β was also voltage-dependent, as demonstrated by the representative traces of Tom40ca-369 blockage by 30 μM pF1β shown in Fig. 8A. Under asymmetric salt conditions, a high negative potential is needed to keep the Tom40 channel open in the presence of pF1β (Fig. 8B).

Unexpectedly, pF1β titrations demonstrated that Tom40 sensitivity to pF1β blockage differed dramatically at different conductive levels. This difference is clearly seen in Fig. 7A; 10 μM pF1β almost completely blocked Tom40ca-369 at L3, induced significant closure of Tom40ca-FL at L2, but had no apparent effect Tom40ca-319 at L1. Although the initial observations illustrated in Fig. 7A could be interpreted as construct-specific binding sensitivity, multiple repeats of the same experiment proved otherwise. Specifically, we found that it was the conformational state of the channel corresponding to a particular conductive level that governed the affinity of pF1β binding. Representative channel recordings demonstrating this level-dependent substrate sensitivity are shown for Tom40ca-332 (Fig. 9). The lowest conductive level, L1, is minimally affected by the addition of 10 μM pF1β (Fig. 9, trace A), whereas the same amount of peptide significantly induces closure of the channel at the higher conductive levels, L3 and L4 (Fig. 9, traces D and E). Unexpectedly, the channel at the L2 conductive level exhibited a “dual behavior” with regard to pF1β sensitivity, with some experiments demonstrating minimal effect as observed for L1, and other experiments revealing a much higher sensitivity on par with L3 (compare traces B and C in Fig. 9). This dual behavior of L2, with both low (L2L) and medium (L2M) sensitivity to pF1β addition, was observed whether or not the N- or C-terminal helices were present.

To quantify the effect of pF1β, and to account for the inherent variability of the open channel probability in the absence of peptide (compare control traces without peptide in Figs. 7 and 9), the probability of observing the channel in the open state, Popen, was calculated in the presence of peptide and normalized over the Popen measured in the absence of peptide for each experiment. Analysis of the normalized probabilities calculated for the four conductive levels of all Tom40 channels at different pF1β concentrations revealed three distinct responses characterized by low, medium, and high peptide sensitivity (Fig. 10A). Each normalized Popen value in this analysis is an average of data obtained at the given pF1β concentrations for all Tom40 constructs. The “low” substrate sensitivity group is composed of L1 and L2L, with an inhibitory concentration (IC50) of 39 ± 3.3 μM. The “medium” sensitivity levels (L2M and L3) gave an estimated IC50 of 2.2 ± 0.1 μM. Finally, the “high” substrate sensitivity level (L4) exhibited an IC50 of 0.4 ± 0.04 μM. The lack of any discernable Popen decrease for any of the conductive levels in the presence of 10 μM SynB2 confirms pF1β-specificity of the channel blockage at every level (Fig. 10B). Taken together, these results indicate that the Tom40 channel has at least three apparent affinities for the presequence peptide and that neither functional channel formation nor presequence interaction requires the first 51 N-terminal amino acid residues or the C-terminal 13 amino acids of Tom40.

To quantitate these presequence binding results we used a simple first-order binding reaction for channel transitions between its open state at level l, O(l), and closed (blocked by peptide) state C(l) as shown in Reaction 1,

$$
\frac{\text{O}^{(l)}}{\text{K}_{\text{off}}^{(l)}} \rightarrow \text{C}^{(l)}
$$

Reaction 1 takes into account our observation that the Tom40 channel returns to the same level upon peptide release (Fig. 6A). The on-rate coefficient is proportional to peptide concentration,
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[pF,β], so that \( K_{\text{on}}^{(l)} = K_{\text{off}}^{(l)} [\text{pF,β}] \), and the concentration-independent off-rate coefficient (the inverse of the average time spent by the peptide in the channel) is \( K_{\text{off}}^{(l)} = K_{\text{off}}^{(l)} \). The on-rate itself is a product of the probability of finding the channel in the open state \( l \) by the corresponding on-rate coefficient, \( j_{\text{on}}^{(l)} = P_{\text{open}}^{(l)} K_{\text{on}}^{(l)} [\text{pF,β}] \), and, because \( P_{\text{closed}}^{(l)} = (1 - P_{\text{open}}^{(l)}) \), the off-rate is \( j_{\text{off}}^{(l)} = (1 - P_{\text{open}}^{(l)}) K_{\text{off}}^{(l)} \). In equilibrium conditions, the on-rate equals the off-rate, \( j_{\text{on}}^{(l)} = j_{\text{off}}^{(l)} \), which gives Equation 1,

\[
P_{\text{open}}^{(l)} = \frac{1}{1 + [\text{pF,β}]/K_d^{(l)}}
\]

(Eq. 1)

\( K_d^{(l)} \) is the dissociation constant (or \( IC_{50} \)) for the peptide interaction with the channel in state \( l \), which is the ratio of the off- and on-rate constants \( K_d^{(l)} = K_{\text{off}}^{(l)}/K_{\text{on}}^{(l)} \). The data in Fig. 10A are fitted according to Equation 1 with \( K_d^{(l)} = 0.4, 2.2, \) and \( 40 \mu M \) for the high, medium, and low sensitivity to peptide binding, respectively.

Discussion

By screening Tom40 proteins from a diverse range of eukaryotes, we have found the Tom40 of the yeast \( C. glabrata \) to be highly amendable to recombinant expression and functional analysis. We have used reconstitution into planar lipid membranes to investigate the channel properties of the Tom40 \( \beta \)-barrel domain, the influence of the N and C termini, and the binding of a mitochondrial targeting presequence to the Tom40 channel. Resolving a controversy in the field (42, 49, 54), we demonstrate that a recombinant Tom40 exhibits at least four distinct conductive levels. Our analyses of these conductive levels reveal for the first time that Tom40 interacts with a model substrate with discrete \( K_d \) values of \( 0.4, 2.2, \) and \( 40 \mu M \). This study therefore suggests that conformational changes in Tom40, as indicated by the transitions between distinct conductive levels, are a means to regulate binding interactions with precursor substrates.

The conductive levels L1 to L4 (≈125–500 pS in a 250/20 mM KCl gradient) correspond well to the conductive states S2 to S5.
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(~120–410 pS) described for Tom40 purified from N. crassa mitochondria, assayed under similar experimental conditions (54). Observing these distinct conductive levels for Tom40ca-FL and nearly identical behavior for the truncated Tom40ca-369, Tom40ca-332, and Tom40ca-319 confirms functional refolding of these proteins. Significantly, it also reveals that a Tom40 lacking a majority of the N-terminal domain can undergo the necessary conformational changes to create the states exhibiting these four conductive levels.

Prior studies on the TOM complex have hinted at considerable Tom40 conformational flexibility, with a rapid “flickering” conductance that may result from a highly flexible Tom40 barrel domain and/or a highly mobile “gating domain” (47, 48, 55). Our observation of up to four discrete conductive levels, showing different apparent affinities for a model substrate, indicates that reconstituted Tom40 adopts distinct conformations even in the absence of the other TOM complex proteins. We hypothesize that the discrete channel conductive levels, with comparable ion permeability ratios but progressively higher ion conductances (Table 1), result from functionally important conformational changes in Tom40. These conformations yield a Tom40 channel with four different affinities to precursor protein substrates, ranging from weak to strong.

The well resolved direct transitions between all four sublevels and the closed state could suggest that a single refolded Tom40 channel is adopting four distinct conductive conformations. Although not impossible, it is difficult to envision a struc-

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**FIGURE 8.** Tom40 blockage by pF1β peptide is voltage-dependent. A, current traces of Tom40ca-369 (L3) at different applied negative voltages as indicated, in the presence of 30 µM pF1β peptide in the cis compartment. These representative traces are from the same channel reconstitution experiment. B, voltage dependence of the open probability ($P_{open}$) of Tom40ca-369 (L3) in the presence of 30 µM pF1β peptide. Dotted line is drawn to aid visualization of the relationship between $P_{open}$ and voltage.

**FIGURE 9.** pF1β peptide blocks Tom40 states of different conductive levels with distinct sensitivity. Current traces of the four conductive levels of Tom40ca-332 before (control, left column) and after addition of 10 µM pF1β peptide (right column). 10 µM pF1β peptide has little effect on L1 (A), moderately blocks L3 (D), and produces sustained blockage of L4 conductance (E). In repeated experiments, L2 demonstrated a sensitivity either similar to L1 (B) or L3 (C). Each control and pF1β addition paired traces are from the same experiment, while recordings of individual conductive levels are from independent experiments. Experimental conditions are as in Fig. 4.
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**FIGURE 10.** Specific binding of pF1β to Tom40 demonstrates three distinct binding sensitivities that correspond to conductive levels. A, averaged open probability ($P_{\text{open}}$) data calculated for Tom40ca-FL, Tom40ca-369, Tom40ca-332, and Tom40ca-319 at increasing concentrations of pF1β. Open probability in the presence of pF1β was normalized against $P_{\text{open}}$ control measurements without peptide. Data are fit to Equation 1 to yield dissociation constants ($K_d$) of 0.4, 2.2, and 40 μM for low sensitivity (L1 and L2), medium sensitivity (L2M and L3), and high sensitivity (L4), respectively. Data points are a mean value of 2–8 independent experiments (± S.E.), obtained from all four Tom40 protein constructs at each of the four conductive levels. Applied potential was 0 mV, and pF1β peptide was added to the cis compartment. B, normalized open probability of Tom40ca-FL and the three mutant Tom40 channels in the presence of 10 μM pF1β or SynB2 peptide. Data are mean values of 2–4 independent experiments, and error bars indicate ± S.E.

**FIGURE 11.** A dimeric model for the Tom40 channel is proposed to explain the four observed conductive levels and three apparent pF1β substrate sensitivities. The observed four incremental increases in channel conductance can be interpreted as paired combinations of two partially or fully open Tom40 pores, and the differential substrate affinity for a particular conductive state can be postulated as resulting from the presence of either partially or fully open channel conformations.

A cultural scenario in which a monomeric pore can adopt four states of different conductance with almost identical cation selectivity and separated by nearly equal conductance increments. Even more difficult to explain is how the L2 conductive level can exhibit two different substrate sensitivities.

We propose instead that the low/medium pF1β-sensitivity dual behavior of the L2 conductive level is more easily understood in the context of a Tom40 dimer, in accord with direct observations of the Tom40 constructs purifying as dimers. A model for such a Tom40 dimer is shown schematically in Fig. 11, in which each circle indicates one Tom40 protomer that can adopt one of three states corresponding to different conductive levels as follows: a closed state (filled circle), a half-open state (half-filled circle), or a fully open state (open circle). We speculate that the observed incremental ∼120-pS increases in conductance represent transitions between these states. To rationalize the same pF1β sensitivity of the L1 and L2L conductive levels, the following two assumptions are necessary: 1) that peptide binding is low ($IC_{50} ∼40 μM$) for a closed protomer and for the half-open pore and 2) that sensitivity to pF1β is high for a fully open Tom40 protomer and increases with the number of fully open pores in the dimer. In the case of two fully open pores (L4), pF1β sensitivity is highest ($IC_{50} ∼0.4 μM$). A combination of Tom40 protomers at low and high sensitivity conformations, whether composed of one fully open and one fully closed pore (L2M) or one fully open and one half-open pore (L3), results in a dimer complex with medium sensitivity to pF1β. This dimer model (Fig. 11) can explain the dual behavior of L2 in terms of substrate sensitivity, where the L2 conductance could be represented by either two half-open pores with low peptide sensitivity (L2L) or by a combination of one closed pore with low sensitivity and one fully open pore with high sensitivity (L2L).

Structural analysis of Tom40 has proven difficult. Recent cross-linking data are consistent with a predicted location for the second N-terminal α-helix inside the Tom40 lumen (56). The location of the first α-helix (removed in the Tom40ca-332 and Tom40ca-319 constructs) has not been characterized. Our data provide new insights into the structure-function relationship of the Tom40 translocase channel. First, amino acids 1–51 and 370–382 of Tom40 are not required for overall pore stability, as Tom40ca-369, Tom40ca-332, and even Tom40ca-319 form stable channels exhibiting four cation-selective conductive levels nearly equivalent to the full-length protein. Second, the so-called “trans binding site,” previously described for presequence binding to the TOM complex (12, 57), is not likely to involve either the N or C terminus of Tom40. The N-terminal deletion removes an evolutionarily conserved α-helix, indicating that this conservation is not driven by interactions with mitochondrial presequences; evolutionary selection for the sequence features in this helix is therefore due to some other aspect of Tom40 function. Finally, it is possible that the N- and/or C-terminal regions of Tom40 form a constriction zone within the channel, which creates a strong electrostatic field. This is suggested by the moderately reduced cation selectivity of L1 for the truncated Tom40 constructs, most notably with permeability ratios of ∼6.6 for Tom40ca-332 and Tom40ca-319 compared with ∼23 and ∼13 for Tom40ca-FL and Tom40wt-FL (Table 1).

Complex conformational changes are consistent with the need for Tom40 to accommodate precursor proteins of various...
sizes and charge profiles, including some that do not carry pre-
sequence peptides, during their translocation through the
TOM complex. Our study adds dynamic aspects to a structural
framework with which to understand the activity of Tom40-
driven protein translocation across the mitochondrial outer
membrane. The extent to which these conformational states
are modified or stabilized by accessory Tom proteins, and
potentially regulated in response to metabolic demands
through phosphorylation of Tom40 and the other Tom pro-
teins (58–60), can now be addressed.

Author Contributions—A. J. K. and S. K. B. initiated the project.
A. J. K. coordinated the study. A. J. K. designed and performed all the
sequence alignments, molecular cloning, and protein engineering as
well the bacterial expression, refolding, purification, and liposome
reconstitution of all Tom40 protein constructs. T. K. R. and D. J.
designed and D. J. performed all electrophysiology and precursor
peptide titration experiments, with analyses by D. J., P. A. G.,
T. K. R., and S. M. B. T. S. constructed additional expression vectors
and performed the mitochondrial membrane experiments of Fig. 2,
which was designed and analyzed by T. S. and T. L. J. M. L. per-
formed the mitochondrial membrane experiments of Fig. 2,
T. K. R., and S. M. B. T. S. constructed additional expression vectors
and performed the mitochondrial membrane experiments of Fig. 2,
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and T. K. R. synthesized all data and wrote the manuscript. T. L.,
D. J., P. A. G., S. M. B., and J. L. contributed to the manuscript writ-
ing. All authors reviewed the results and approved the final version of
the manuscript.

Acknowledgments—Peptide synthesis and validation was expertly
performed by Dr. Galina Abdoulaeva at the Facility for Biotechnology
Resources at the Food and Drug Administration, Center for Biologics
Evaluation and Research. We acknowledge the expert biophysical
analysis assistance by Dr. Rodolfo Ghirlando (Laboratory of Molecu-
lar Biology, NIDDK, National Institutes of Health) and by Dr. Gregorz
Piszczek (Biophysics Center, NHLBI, National Institutes of Health),
and we thank Dr. Matthew Belousoff (Monash University) for critical
comments on the manuscript.

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