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Conserved Substrate-binding by Chaperones in the Bacterial Periplasm and the Mitochondrial Intermembrane Space

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Page Heading: *SurA and TIM10 share substrate binding specificity*

Key words: mitochondria, chaperones, small Tims, SurA, protein translocation, evolution

*SurA and TIM10 share substrate binding specificity***Synopsis**

Mitochondria were derived from intracellular bacteria and the mitochondrial intermembrane space is topologically equivalent to the bacterial periplasm. Both compartments contain ATP-independent chaperones involved in transport of hydrophobic membrane proteins. The mitochondrial TIM10 complex and the periplasmic chaperone SurA were examined in terms of evolutionary relation, structural similarity, substrate binding specificity and function in transporting polypeptides for insertion into membranes. The two chaperones are evolutionarily unrelated; structurally, they are also distinct both in their characteristics as determined by small angle X-ray scattering (SAXS) and in pairwise structural comparison using the distance matrix alignment (DALI Lite server). Despite their structural differences, SurA and the TIM10 complex share a common binding specificity both in pepscan assays of substrate proteins. Comprehensive analysis of the binding on a total of 1407 immobilised 13mer peptides revealed that the TIM10 complex, like SurA, does not bind hydrophobic peptides generally, but that both chaperones display selectivity for peptides rich in aromatic residues and with net positive charge. This common binding specificity was not sufficient for SurA to completely replace TIM10 in yeast cells *in vivo*. In yeast cells lacking TIM10, when SurA is targeted to the intermembrane space of mitochondria it binds translocating substrate proteins, but fails to complete transfer of the substrate to the translocase in the mitochondrial inner membrane. We suggest that SurA was incapable of presenting substrates effectively to the primitive TOM and TIM complexes in early mitochondria, and was replaced by the more effective small Tim chaperone.

INTRODUCTION

Mitochondria and α -proteobacteria share an ancestry, with mitochondria derived from endosymbiotic eubacteria that invaded the first eukaryotic cells over a billion years ago. In bacteria, β -barrel proteins are assembled in the outer membrane by the Omp85 protein translocase, and many α -helical transmembrane proteins are assembled in the inner membrane by the SecY translocase and YidC [1, 2]. Mitochondria have retained types of these membrane translocases: Sam50, the core of the sorting and assembly machinery (SAM) complex is a member of the Omp85 protein family, and Oxa1 and Oxa2 in the mitochondrial inner membrane are derived from the bacterial YidC.

Protein synthesis occurs in the cytoplasm of bacteria and membrane proteins are transferred to the SecYEG complex in the inner membrane. Inner membrane proteins are then assembled with assistance from YidC, while β -barrel protein substrates exit into the periplasm and are transferred to the Omp85 complex in the outer membrane. In *Escherichia coli*, delivery of β -barrel proteins to the outer membrane relies on periplasmic chaperones. Two of these, SurA and Skp, have been shown in various studies to be involved in outer membrane protein maturation [3-9] and while other chaperones (e.g. PpiD, FkpA, DegP) have also been implicated, evidence for the precise role of each is inconclusive [10, 11].

SurA from *E. coli* has been purified and crystallized and consists of four domains: an N-terminal domain, a short C-terminal and two rotamase domains [3, 12, 13]. Neither of the two rotamase domains is required for SurA chaperone activity, a concept supported by several studies. First, a proteolytically stable N-terminal fragment, consisting of the first 150 residues of SurA without any rotamase domain retains the capacity to bind substrate peptides [14]. Second, a recombinant form of the protein, SurA^{NCT}, which has the N-terminal domain fused to the short C-terminal tail but lacking both rotamase domains, shows full chaperone activity *in vitro* and complements the *in vivo* defects of *surA*⁻ mutant [12,15]. All work to date has focussed on SurA from the γ -proteobacterium *E. coli*, and it is unclear whether homologous proteins are found in the periplasm of other groups of bacteria. Thus it remains open what the ancestral condition was in the bacteria from which

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mitochondria were derived, and whether a SurA protein is found in the intermembrane space of mitochondria in any species of eukaryote.

The insertion and assembly of proteins into both the outer and inner membranes of mitochondria is assisted by the small Tim chaperones. Small Tim proteins belong to one of four sub-families, represented by the yeast proteins Tim9, Tim10, Tim8 and Tim13 [16,17]. All small Tim proteins are located in the mitochondrial intermembrane space, and in some organisms a variant form of one of the small Tim proteins (eg Tim12 in yeast) is attached to the inner membrane [17-20]. Two distinct hexameric assemblies, a TIM10 complex (TIM10c) consisting of three Tim9 subunits and three Tim10 subunits, and a TIM13 complex (TIM13c) consisting of three Tim8 subunits and three Tim13 subunits, assist passage of β -barrel protein substrates like porin and Tom40 to the SAM complex in the mitochondrial outer membrane. Both TIM10c and TIM13c also assist inner membrane substrates like the ADP/ATP carrier (AAC) destined for the inner mitochondrial membrane.

We sought to address the relationship between SurA and the small Tim proteins in terms of (i) an overlap in their ancestry, (ii) their structure, (iii) their binding specificity and (iv) their function in membrane protein translocation. A hidden Markov model search identified SurA homologs in a broad range of bacteria, but show that the SurA in many α -proteobacteria lack one or both rotamase domains, providing natural examples of the experimentally-determined minimal functional unit SurA^{N_Ct}. We used solution X-ray scattering (SAXS) to determine a low resolution three-dimensional structure of SurA^{N_Ct}. Despite deletion of the two rotamase domains, SurA^{N_Ct} and SurA show a very similar structural architecture of the N- and C-terminal domains. While both TIM10c and SurA^{N_Ct} are composed of α -helices and loops, they display no obvious structural similarity. Detailed analysis of the substrate-binding of TIM10c, in comparison to that of SurA, revealed that the chaperones share a common binding function, governed by two specific sequence determinants of the substrate: (i) a relatively high content of aromatic residues and (ii) a net positive charge. While bacterial SurA can bind mitochondrial membrane protein substrates *in vitro* with specificity similar to that of TIM10c, it cannot substitute for TIM10c function *in vivo*. TIM10c therefore mediates a further, essential function, apart from its chaperone

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activity, necessary for correct transfer of substrates to downstream translocation machineries.

Stage 2(a) POST-PRINT

EXPERIMENTAL

Hidden Markov models

BLAST searches were used to gather a set of sequences from which to construct hidden Markov models (HMMs), which in turn were used to search the UniProt database for related proteins. The HMMs were built with the program HMMER 2.3.2. The best multiple alignment for each family of sequences was obtained with ClustalW and t-coffee. The two alignment programs produced different best alignments, and we built two HMMs (corresponding to ClustalW and t-coffee alignments) for the SurA family of sequences. The resulting HMMs were used to scan UniProt database release 7.2 (Swiss-Prot release 49.2 and TrEMBL release 32.2) as previously described [21]. The results of HMM searches were manually examined. The sequences used to construct the HMMs were detected from within the UniProt search with E-value scores $< 10^{-40}$.

Apart from the proteins reported here as members of the SurA family, two other protein families were detected with the SurA HMM. One was the bacterial PpiD family (e.g. *Escherichia coli* PpiD, E-value of 9×10^{-11}), periplasmic proteins with functions that overlap those of SurA [11, 22]. The second family, and the only eukaryotic proteins returned in this HMM search, matched only poorly (E-values of $\sim 10^{-04}$ to 10^{-03}) to the model and represents a group of small nuclear proteins consisting simply of a single PPIase domain (eg the human NimA-interacting protein, and yeast protein Ess1). There is no evidence to suggest a relationship between these small nuclear proteins and the bacterial SurA's apart from the presence of a rotamase domain.

Gene mapping

Searches with TBLASTN were used to map the genes identified as *surA* onto the chromosomal sequence from the various species of α -proteobacteria held at the TIGR Comprehensive Microbial Resource (<http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi>), using the Region View tools.

Cloning of SurA variants

SurA and SurA^{Nct} (residues 1-172 and 387-428) were cloned in pET28a (Novagen) for bacterial expression with a C-terminal His₆-tag after PCR amplification from *E.*

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coli genomic DNA using primers SurA-F (GGAATTCCATGGCCATGAAGAAGCTGGAAAAC) and SurA-R-NoStop (ATAAGAATGCGGCCGCGTTGCTCAGGATTTAAC) for SurA; SurA-F and SurAN-R (CCCAAGCTTGCGTCGTTTTGGTTACCC) for the N-terminal domain of SurA; SurACt-F (CCCAAGCTTCGATAAAACCGACGCTGCG) and SurA-R-NoStop for the C-terminal tail of SurA. The sequence encoding the first 80 amino acids of cytochrome b2 (Cyb2), which constitutes its IMS-targeting sequence, were then cloned upstream of SurA^{Nct} in pET28. The Cyb2 targeting sequence was amplified from pSM4 (a gift from Ben Glick, University of Chicago) by PCR using primers Cyb2-F (CGGGATCCATGGGTATGCTAAAATACAAACC) and Cyb2-80-R (CATGCCATGGGGTTGTCTATTTGGCC). For expression in yeast, the whole Cyb2-SurA^{Nct} cassette was cloned into pRS316UP10 using primers Cyb2-F and SurA-R (CCCAAGCTTGCGTCGTTTTGGTTACCC)

In vitro pull-down of radiolabelled proteins

2.5 nmoles of purified His-tagged protein was incubated with 5 μ l translation mixture of the radiolabelled substrate for 30 min at 4°C. 50 μ l of 20 % Ni-NTA slurry in PD buffer (50 mM Tris-HCl pH7.4, 250 mM NaCl, 0.1% BSA, 1% Tween-20, 50 mM imidazole) was added to each protein mixture and incubated with shaking for a further 30 min at 4°C. Samples were centrifuged for 2 min at 3000 g, the unbound material was removed, and the Ni-NTA was washed 2 x 10 min at 4°C with PD buffer. 5 μ l of 6x SB was added to the resin and the residual buffer, and samples were boiled for 3 min at 95°C then vortexed for 10 sec, boiled again and vortexed again, before loading on SDS-PAGE. Gels were stained with Coomassie, dried, exposed overnight onto a Fuji imaging plate, and visualised with a Storm 840 scanner (Molecular Probes). The intensity of each band was quantified using AIDA software.

Solution X-ray Scattering

Scattering data were collected at station 2.1 of the U.K. Synchrotron Radiation Source (SRS) Daresbury [23] equipped with a multiwire gas detector [24]. Samples were measured at concentrations between 0.5 and 5 mg/ml covering a momentum transfer range of $0.02 \text{ \AA}^{-1} < q < 0.4 \text{ \AA}^{-1}$ with $q = 4\pi \sin \Theta / \lambda$ (where 2Θ is the scattering angle and λ the X-ray wavelength of 1.54 Å). Data collection and treatment were performed

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as described previously [25]. In addition the program BUNCH [26] was applied using the known high resolution SurA structure [13] in order to obtain a model for the SurA^{N^Ct} construct formed by N- and C-terminal domain residues (25-165 and 395-427, SurA residue numbering) only i.e. without the two rotamase domains, but connected by additional 17 residues. Specifically, the structure of the N-terminal segment (res.25-150) as well as the C-terminal β -strand (res.422-427), preserving the characteristic β -sheet, was kept fixed. Besides, the two α -helices (res.155-163 and res.395-420) were maintained but allowed to move in the overall structure to permit the accommodation of the polypeptide segment linking N- and C-terminal domains.

Miscellaneous

The wild-type yeast strain W303, P_{MET3}-TIM10 [27] and Tim9-19 [28] were used. Purification of mitochondria, *in vitro* imports, purification of His-tagged fusion proteins, yeast growth complementation in P_{MET3}-TIM10 and screening of peptide scans were performed according to published methods [27,29-31]. Peptide hydrophobicity was calculated using the peptide library design and calculator tool (Sigma Aldrich). Hydropathy values assigned to each peptide predict the solubility characteristic in an aqueous solution. Values are based on known hydropathy values per amino acid, divided by the length of the peptide sequence, on a scale of -1 (very hydrophilic) to 2.25 (very hydrophobic).

RESULTS

SurA is present in α -proteobacteria, that share ancestry with mitochondria

The crystal structure of SurA from *E. coli* [13] shows four domains, summarized in Figure 1a. Proteins homologous to the SurA from *E. coli* were readily identified in species of β -, γ - and δ -proteobacteria (see Methods). A hidden Markov model describing the SurA protein family was constructed from these sequences and used to search the UniProt data set, revealing SurA sequences in many other groups of bacteria. E-value scores in the region of 10^{-90} to 10^{-200} were typically retrieved: protein sequences with E-value scores less than 10^{-07} were often small proteins, containing only a rotamase domain, and this was taken as a cut-off for the SurA family. Candidate SurA proteins were found in the five groups of proteobacteria ($\alpha, \beta, \gamma, \delta, \epsilon$) as well as divergent groups such as the Chlorobiaceae, and in further species including *Deinococcus radiodurans* and *Cytophaga hutchinsonii* (Figure 1b). Many SurA sequences found in α -proteobacteria, including *Rickettsia* which represents the closest relative to the mitochondrial ancestor, share a conserved N-terminal domain but have no rotamase domains. These then represent natural examples of the SurA^{NCT} chaperone.

To independently determine whether the α -proteobacterial proteins detected with the HMMs are homologs of SurA, gene synteny was analysed. In *E. coli*, the *surA* gene sits in a cluster of genes ordered to encode: OstA, SurA, PdxA and KsgA. In α -proteobacteria too, the *surA* gene sits between those encoding the homologs to OstA and KsgA (Table 1). The presence of a SurA^{NCT}-type chaperone in many species of α -proteobacteria lends independent verification that this form of SurA can function as a *bona fide* chaperone.

Structural comparison between SurA and TIM10c

Since the rotamase domains of SurA are not present in TIM10c, and furthermore are not required for SurA function, we used SurA^{NCT} for our structural studies, assuming that inclusion of the rotamase domains would increase the chance of obtaining a false-negative result. We used small angle X-ray scattering (SAXS) to produce a molecular envelope for recombinant SurA^{NCT} (Figure 2a). The restored shape suggests SurA^{NCT} to be an elongated particle with overall dimensions of approximately 90Å x 45Å x

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40Å. Then, the program BUNCH [26] was used to reconstruct a model for SurA^{NCt}, including in particular the connection between N- and C-terminal parts as a result of the missing rotamase domains in our construct, based on the crystal structure published for SurA [9]. This enhanced structure was used to determine a theoretical scattering profile for SurA^{NCt}, which gave a very good fit to the experimentally determined scattering data for SurA^{NCt} (Figure 2b). The BUNCH model suggests a change in the orientation of the long C-terminal helix (corresponding to residues 395 to 421 of full-length SurA) and the short helix (residues 155 to 163) at the end of the N-terminal chaperone domain. Nevertheless, deletion of the two rotamase domains does not seem to perturb the arrangement of secondary structure elements within the N-terminal and C-terminal domains of the SurA chaperone domain.

The secondary structure elements present in TIM10c [25, 32] are similar to those in SurA^{NCt} [13], both proteins comprising short α -helices and loops. The shapes of the TIM10c and Tim9 and Tim10 subunits from yeast have been described using small angle X-ray scattering (SAXS) [25]. The structural parameters radius of gyration (R_g) and maximum dimension (D_{max}) experimentally determined for SurA^{NCt} are clearly different to those reported earlier for TIM10c or the individual Tim9 and Tim10 subunits (Table 2). Furthermore, the SurA^{NCt} scattering profile was compared with that of TIM10c, and with both individual subunits, but none of these show similar scattering characteristics (Figure 2c) clearly highlighting the difference in tertiary and quaternary structure of these proteins. SurA^{NCt} is a monomer in solution with a size of about 19 kDa (data not shown) and much smaller than the ~60 kDa hexameric TIM10c; therefore, considering the structural parameters of Table 2, SurA^{NCt} is a more extended particle than TIM10c, and bears limited structural resemblance to it.

The DALILite server can be used to assess topological similarities between pairs of evolutionarily unrelated proteins [33, 34]. For relevant examples, alignments with the archaeal cytosolic chaperone prefoldin (PFD), the bacterial periplasmic chaperone Skp and the bacterial cytosolic chaperone trigger factor (TF) were calculated. The experimentally determined minimal chaperone domain of trigger factor [35] was recently reported to bear a striking resemblance to SurA^{NCt} [36]. Pairwise alignment of SurA^{NCt} with TIM10c generated a low Z-score, below the significance threshold of 3, with both proteins scoring better against other chaperones than against each other

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(Table 1) [37]; SurA^{NCT} was most similar to the chaperone domain of Trigger factor (Z-score 6.2), while TIM10c was most similar to prefoldin (Z-score 3.5). Based on the above combined data we therefore conclude that TIM10c does not share a high degree of structural similarity with SurA^{NCT}.

SurA mimics TIM10 complex binding to mitochondrial substrates

To determine whether SurA could bind mitochondrial substrate proteins, porin, Tom40 (two outer membrane proteins that are predicted to fold in β -barrels in the outer membrane), AAC (an inner membrane protein that consists of 6 α -helical transmembrane segments [38]) and Su9-DHFR (a matrix-targeted fusion between residues 1-69 of ATPase subunit 9 and mouse dihydrofolate reductase) were synthesised *in vitro* and presented to SurA or TIM10c in pull-down assays (Figure 3). For all three membrane proteins, levels of precursor binding were comparable for SurA and for TIM10c, both being well above background levels. The control protein Su9-DHFR, which is not a substrate of TIM10c, did not bind to either chaperone.

This *in vitro* assay gives only limited information on substrate binding, since we cannot control the stability of pre-formed complexes between precursors and chaperones present in the reticulocyte lysate. In addition, if specific conformational signals in the translocating precursor contribute to binding of TIM10c or SurA, these are not necessarily recreated in this assay. To better compare TIM10c and SurA, we used pepscan assays that enable binding specificities to be quantified. A cellulose membrane carrying peptides representing the mitochondrial inner membrane protein AAC or Tim22 was screened with purified SurA or TIM10c, and binding patterns were analysed (Figure 4). These demonstrate that both chaperones bind to specific sets of discrete, discontinuous peptide spots. There is a large degree of overlap in the peptides preferred by the two chaperones.

TIM10c and SurA prefer similar peptide substrates

To dissect further the substrate specificity of TIM10c and TIM13c, peptide scans of AAC, Tim22, Porin and Tom40 were probed. Although the peptide spots bound by a particular chaperone generally correspond to the same region of the substrate, the exact spots which are bound can vary somewhat between experiments. For this reason

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data were collected from a number of experiments, and analysed statistically. The data for binding of members of the small Tim family (TIM10c, Tim10, TIM13c and Tim13) from a total of fifteen pepscan membranes were analysed. This equates to a total number of 1407 data points. The intensity of each spot was quantified and classed as bound or non-bound, depending on the spot intensity. According to this classification, of the 1407 data points, 336 were “bound”, and 1071 were “non-bound”. The substrate-binding capacity of SurA has previously been studied in detail, by testing its binding to pepscan membranes representing bacterial substrate proteins [39].

Analysis of the amino-acid distribution in bound peptides, compared to the library as a whole, is shown in Figure 5. As was demonstrated for SurA, peptides bound by the small Tims are highly enriched in aromatic amino acids (Phe, Trp and Tyr). This preference is unlikely to reflect non-specific hydrophobic interactions since none of the hydrophobic amino acids isoleucine, valine or alanine shows a clear association with binding. SurA, too, binds preferentially to peptide substrates carrying aromatic residues [39]. Although histidine, which was enriched in SurA-binding peptides, is not shown to be favoured here, the occurrence of His-containing peptides in the data set was just 0.057%, the lowest of any amino acid, so this result may not be significant. A χ^2 test to determine if the calculated associations are significant was performed, and bars for amino acids which showed a significant association with bound or non-bound peptides ($p < 0.05$) are coloured dark grey (Figure 5a). Of the 336 bound peptides, only 7% lacked aromatic amino acids, while 69% contained two or more aromatic residues. Classification of the peptides according to their aromatic content revealed that the fraction of the group bound by the small Tims increases with the number of aromatic residues (Figure 5b). Since aromatic amino acids are large, hydrophobic residues, it may be argued that this observed preference is a product of a more general preference for hydrophobic peptides. However, classification of the peptides according to their hydrophobicity showed some preference for more hydrophobic peptides, but no distinct relationship was observed. When the average hydrophobicity of bound and non-bound peptides was compared for groups of peptides with the same aromatic content (Figure 5c), only a slight increase in hydrophobicity was observed for bound peptides, and for the 337 peptides which contain no aromatic residues, the average hydrophobicity of bound peptides was even

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slightly lower than that of non-bound peptides. We therefore conclude that, like SurA, the small Tims display a specific preference for binding peptides enriched in aromatic amino acids, which is greater than their general preference for hydrophobic peptides.

The effect of each peptide's net charge on small Tim binding was also investigated. The relative occurrence of glutamate residues in binding peptides was reduced for both the small Tims and for SurA. Classification of the peptides according solely to their net charge revealed no distinct pattern in bound versus non-bound peptides (Figure 5b). However, plotting the average net charge of SurA-binding and SurA-nonbinding peptides as a function of the number of aromatic amino acids in the peptides [39] revealed the average net charge of SurA binding peptides in each group (where each group is composed of peptides with identical aromatic amino acid content) was +0.6 charge units higher than that of the SurA non-binders. The same analysis was carried out for small Tim binding peptides (Figure 5c). While the magnitude in net charge between bound and non-bound peptides was less for the small Tims (+0.1 to +0.4 charge units) than for SurA (+0.6 charge units), the trend was the same *i.e.*, for peptides with identical aromatic amino acid content, the average charge of those bound by small Tims or by SurA is more positive than the average charge of those not bound. This reveals that the peptides' electrical charge does have bearing on their capacity to bind the chaperones, but in a random set of peptides this effect is masked by the much larger influence of aromatic amino acids.

Function complementation: Can SurA complement for TIM10 function?

The above analysis established an overlap in the substrate binding capacity of SurA and TIM10c. However, *in vivo*, TIM10c must collect substrates from the TOM complex and deliver them to the outer and inner membrane insertion complexes. We therefore addressed whether SurA is capable of rescuing a yeast strain lacking TIM10c, and whether it can complement TIM10c function in protein translocation. Since our structural data are based on the truncated SurA^{N_Ct}, and biochemical data have shown this complements bacterial *surA* mutants *in vivo*, we used this variant for complementation studies. SurA^{N_Ct} is imported into purified mitochondria at low levels (Figure 5b). To increase its import, the targeting signal of the intermembrane space protein cytochrome b2 was fused to the amino-terminus of SurA^{N_Ct}. The SurA

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variants used, their import and localisation in purified mitochondria are shown in Figure 6 (panels A and B).

A yeast strain where the *TIM10* gene is under the regulation of the methionine-repressible MET3 promoter was used. Addition of 0.2 mM methionine to the growth medium results in repression of the essential *TIM10* gene [27]. Growth of these cells was compared with the same strain transformed with a plasmid carrying the intermembrane space-targeted pSurA^{Nct}, or with the same vector carrying *TIM10* (Figure 6c). While *TIM10* fully restored growth on +Met medium, SurA^{Nct} did not complement growth. Failure of SurA to interact properly with one or a few essential substrate proteins, and/or with other essential components of the mitochondrial import apparatus, might explain why SurA cannot complement the loss of TIM10c.

To specifically address whether SurA^{Nct} can restore import of AAC into purified *tim9-ts* mitochondria, which lack the TIM10 complex (Figure 6d), radiolabelled AAC was imported into *tim9-ts* mitochondria, into which either Tim10, SurA^{Nct} or buffer only had been pre-imported. Tim10 was included as a positive control, since previous experiments have demonstrated its ability to partially restore AAC import into mitochondria lacking TIM10c [29]. After import of AAC, re-isolated mitochondria were treated with low (25 µg/ml) or high (200 µg/ml) levels of proteinase K. This was to distinguish between AAC at stage IIIa of import, still attached to the TOM channel and susceptible to low proteinase K, stage IIIb AAC which has been removed from the TOM channel and is now resistant to low proteinase K, and stage IV AAC which has been inserted into the inner membrane and is resistant to high proteinase K.

In all cases, a small amount of AAC is visible after treatment with low proteinase K, i.e. fully translocated across the TOM channel independently of the presence of TIM10c. A fraction of this was still visible after high proteinase treatment, which represents the fraction which has been inserted into the inner membrane. A small amount of AAC is inserted into the inner membrane with no pre-import (lane 6), and this amount was increased by pre-import of Tim10 (lane 2). Tim10 also increased the total amount of import across the OM (lane 1). For the mitochondria with SurA^{Nct} pre-import, the amount of intact AAC remaining after low proteinase K treatment was the same as for mitochondria with no pre-import (lanes 3 and 5). An extra fragment

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corresponding in size (~10kDa) to one module of AAC was observed in the low proteinase K sample at around 10 kDa (denoted by *). This suggests that an extra part of the AAC molecule is able to reach a low proteinase K-protected location when SurA^{Nct} is present in the intermembrane space; SurA might facilitate partial AAC entry into the TOM complex, or this module may resist proteolysis because it is contacting and protected by SurA^{Nct}. However, this partially translocated AAC is a “dead-end” intermediate which is not translocated further.

DISCUSSION

The role of chaperones in protein translocation

A general chaperone activity has been demonstrated using heterologous substrates for both TIM10c and SurA [12,30]. The natural substrates of these chaperones – α -helical membrane proteins of the mitochondrial carrier family, and outer membrane proteins which fold into β -barrels – contain many hydrophobic amino acids. Although it is not clear what level of secondary structure is present in substrate preproteins during translocation, some (if not all) hydrophobic residues must be exposed during transit through the TOM or SecYEG translocation channels. A chaperone activity is therefore required to collect the preproteins as they exit the channel, and to prevent preprotein aggregation. For ATP-independent chaperones, this activity appears to be mediated by a dynamic interaction between chaperone and substrate, preventing off-pathway interactions, but being displaced by correct downstream interactions [40,41].

TIM10c and SurA have a common binding specificity. Both are able to bind to short peptides, and both exhibit a preference for aromatic and positively charged residues. SurA at least partially substitutes the chaperone function of TIM10c in AAC translocation, as shown by an interaction between translocating AAC and SurA^{Nct} in the intermembrane space (Figure 6d). Since TIM10 and SurA are both able to function as a general chaperone on heterologous substrates [12,30], the specificity of the translocation reaction is probably at the level of targeting or transfer, not of substrate-binding. That SurA is able to bind the mitochondrial substrate AAC *in organello*, as well as *in vitro*, adds weight to the argument that the chaperone functions of SurA and TIM10c are equivalent, and that both are capable of binding proteins that are helical in structure. However, this chaperone activity is sufficient neither for full translocation of AAC, nor for restoring growth to TIM10-depleted cells.

Since neither TOM nor TIM22 has a prokaryotic homologue it is a logical inference that, while SurA may be able to “chaperone” translocating preproteins, it is inefficient in their release from the TOM complex and/or transfer to the TIM22 complex. The SAM complex, however, does have a prokaryotic homologue, Omp85. The question

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of whether SurA is able to assist transfer of β -barrel precursors from TOM to SAM remains open. We were unable to test this in our assay, because of a redundancy in the chaperones mediating β -barrel translocation in mitochondria: Tim9-*ts* mitochondria can translocate β -barrel substrates as they still contain the TIM13 complex which also functions in this pathway.

Binding of membrane protein substrates by SurA and the small Tim chaperones

SurA recognizes substrate polypeptides by virtue of short hydrophobic segments, particularly sequences rich in aromatic residues [9,15,39]. In the crystal structure of SurA an interaction with a peptide loop from a neighboring SurA molecule is observed, defining the likely substrate-binding groove. Surprisingly, this "substrate" peptide sits in an α -helical conformation [13,42]. While the mature β -barrels have little if any helical structure, the unfolded substrate polypeptides might assume short, non-native helical structures in the context of the chaperone. The substrate may take on extensive β -structure only on release to the lipid environment and Omp85 complex in the outer membrane [43,44].

The predominant substrates carried by the small Tims are the abundant carrier proteins, where the mature protein in the inner membrane consists of $\sim 70\%$ α -helix [38], and the segments of AAC that will form the α -helical transmembrane domain are the regions of the protein bound most avidly by TIM10c [31,46,46]. If β -barrel substrates can be induced to take on short helical conformation prior to membrane integration, it would explain how the binding crevices of TIM10c and TIM13c chaperones can carry both β -barrel and α -helical transmembrane protein substrates.

Functionally-related chaperones in eukaryotes and prokaryotes

We find no evidence for an evolutionary relationship between SurA and the small Tims. Furthermore, we find no obvious common structural architecture in the two chaperones, beyond the level of their secondary structure. While TIM10c and SurA share the same molecular chaperone function, it seems that substrate release by each of the chaperones is a specialized process. SurA is thought to dock and deliver relatively unfolded substrates directly and specifically to the Omp85 complex in the bacterial outer membrane [44,47-49]. We suggest that SurA is

SurA and TIM10 share substrate binding specificity

ineffective at release of substrates like AAC to the TIM22 complex in the mitochondrial inner membrane, and that this is relevant in explaining the development of the small Tim chaperones in the course of transforming intracellular symbionts to mitochondria.

Carrier molecules such as AAC are found only in the intracellular membranes of eukaryotes and are likely to have been derived as a means to enslave the endosymbiont that gave rise to mitochondria [50-52]. The efflux of ATP and other metabolites the carriers provide to the host cell represent a rich benefit to drive improvements to the protein import pathway required to install these carriers in the mitochondrial inner membrane. A primitive TOM complex [53,54] and the pre-existing translocases YidC and SecYEG [55] may have provided for ineffective but sufficient import and assembly of early carrier molecules [52]. Some inefficient import of inner membrane proteins in the absence of small Tim chaperones is tenable; no small Tims are found in eukaryotes such as microsporidians where their reduced mitochondrial proteome means that vestigial TOM, SAM and TIM complexes can operate to assemble membrane proteins in the absence of chaperones in the intermembrane space.

It cannot be known whether or not SurA was present in the periplasm of the endosymbiont. However, the emergent need to transfer carrier molecules from the outer membrane TOM complex to the inner membrane translocases is a scenario in which development of a small TIM chaperone would provide a selective advantage, and recent sequence analysis of the small Tim family suggests that a single, Tim10-like protein, would be sufficient to function as a chaperone for carriers [17]. Gene duplications and specializations would readily provide the Tim9, Tim8 and Tim13 subunits from the initial Tim10. The use of SurA could have been discontinued anytime after the small Tims were installed, given these same chaperones can deliver substrates to both the outer (SAM) and inner (TIM) complexes.

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SurA and TIM10 share substrate binding specificity

species	genome size (Mb)	<i>ostA</i>	<i>surA</i>	<i>pdxA</i>	<i>ksgA</i>
<i>Escherichia</i>	5.23	Q8CWE6 60301-57947	P21202 57894-56608	Q8FL95 56608-55619	Q8FL96 55622-54801
<i>Nitrobacter</i>	3.40	Q3SS01 1830344-1832833	Q3SS00 1833024-1833968	Q3SRZ9 1834071-1835078	Q3SRZ8 1835075-1835938
<i>Brucella</i>	3.29	Q8YG96 1312437-1314830	Q8YG95 1315161-1316114	P58712 1315999-1317210	Q8YG94 1317207-1318037
<i>Zymomonas</i>	2.05	Q5NMX5 1325487-1327778	Q5NMX4 1327897-1329312	Q5NMX3 1329354-1330361	Q5NMX2 1330372-1331208
<i>Bartonella</i>	1.93	Q6G436 631997-629628	Q6G437 629544-628576	Q3V7R0 628576-627563	Q6G438 627572-626742
<i>Rickettsia</i>	1.11	O05950 838042-835907	O05951 835723-834836	none	O05952 834710-833904

Table 1. Gene positions for *Escherichia coli* and five species of α -proteobacteria. The species used are *Escherichia coli*, *Nitrobacter winogradskyi*, *Brucella melitensis*, *Zymomonas mobilis*, *Bartonella henselae*, *Rickettsia prowazekii*. The SwissProt code of the corresponding protein is given for each gene and the numbers refer to the position of the gene on the genome map at the Comprehensive Microbial Resource (<http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi>).

Protein	Radius of gyration R_g (Å)	Maximum dimension D_{max} (Å)	Reference
SurA ^{NCT}	28.8 ± 0.1	95 ± 5	This study
Tim10 subunit	21.9 ± 0.2	68 ± 2	25
Tim9 subunit	22.3 ± 0.2	71 ± 2	25
TIM10c	27.7 ± 0.2	85 ± 3	25

Table 2. Structural parameters measured by small angle X-ray scattering (SAXS) for the corresponding proteins as indicated

SurA and TIM10 share substrate binding specificity

	TIM10c	SurA^{Nct}	Skp	PFD	TF C-ter
TIM10c		2.5	3.1	3.5	2.2
SurA^{Nct}	2.5		2.5	2.4	6.2
Skp	3.1	2.5		8.2	2.4
PFD	3.5	2.4	8.2		3.2
TF C-ter	2.2	6.2	2.4	3.2	

Table 3. Z-scores for DALILite pairwise structural alignments. Scores below 3 are not considered significant. TIM10c: *S. cerevisiae* TIM10 complex (PDB 2BSK). SurA^{Nct}: *E. coli* SurA chaperone domain (from PDB 1M5Y). Skp: *E. coli* Skp (PDB 1SG2). PFD: *M. thermoautotrophicum* prefoldin (PDB 1FXK). TF C-ter: *E. coli* trigger factor chaperone domain (from PDB 1W26).

FIGURE LEGENDS**Figure 1 SurA is found in diverse groups of bacteria**

(a) Domain organization of SurA from *Escherichia coli* (PDB code 1m5y [9]). In one view, the two rotamase domains are colored green; these have been deleted in the second view to emphasize the arrangement of the N-terminal (blue) and C-terminal (red) domains of SurA.

(b) The domain structure of the SurA proteins from various bacteria are shown compared to the well-characterized SurA from *E. coli*. The black box represents the predicted signal sequence (Signal P 3.0), the N- and C-terminal domains of the *E. coli* protein are shown in blue and red respectively, and the green boxes show regions conforming to rotamase (PPIase) domains. Species represented below the dotted line are members of the α -proteobacteria. The N-terminal domain is a defining feature of the entire SurA protein family, with the C-terminal extension being less well-conserved in α -proteobacteria.

Figure 2 Structure of SurA^{NCt}

(a) Three orthogonal semi-transparent surface representations (shown as assembly of grey spheres) of SurA^{NCt}. This shape is an average computed from 12 shape reconstructions that have been deduced from SAXS. A ribbon drawing of a BUNCH model is superimposed on each shape orientation. N- and C-terminal domains are highlighted in blue and red colour, respectively. The green segment highlights the fragment linking N- and C-terminal parts. The first two views correspond to views of the full SurA chain shown in Figure 1a. The molecular graphics software PyMol was used (<http://pymol.sourceforge.net>).

(b) Comparison of the experimental X-ray scattering profile of SurA^{NCt} (red error bars) with the scattering profile of a structural model obtained with the program BUNCH (blue curve; goodness-of-fit value $\chi = 1.5$ and depicted in A) containing all residues consistent with our SurA^{NCt} construct. The dark red curve corresponds to the fit from the shape reconstruction (shown in A).

(c) Experimental scattering profiles of SurA^{NCt} monomer (red), Tim10 dimer (green) and TIM10c hexamer (blue). The solid curves underline the different scattering features and are the result from shapes fitted to each curve (see panel a and ref.18).

All curves were normalized to unity at zero scattering angle.

Figure 3 *In vitro* binding of SurA and TIM10c to mitochondrial membrane proteins

Radiolabelled Su9-DHFR, Tom40, AAC and Porin were synthesised by *in vitro* coupled transcription/translation. These were then incubated with SurA-His, His-TIM10c, or no chaperone ('control') and precipitated with Ni-NTA. 10% of the total radioactive material used was loaded for quantification purposes. Signals were analysed by digital autoradiography, quantified, and adjusted to the level of the 10% standard.

Figure 4 Binding of SurA and TIM10c to peptide libraries

Peptide libraries for Tim22 (left) or AAC (right) were screened with purified TIM10 complex or SurA. Bound protein was transferred to PVDF and detected with the appropriate antibodies. Immunoblots are shown alongside a graphical representation of the relative amount of binding to each peptide spot. The positions of predicted transmembrane domains and loops, according to peptide number, are indicated below the graphs.

Figure 5 Substrate specificity of the small Tim family

(a) The relative occurrence of each amino acid in small Tim-binding peptides was normalised to its occurrence in the whole peptide libraries (=100%). A graph showing the relative amino acid occurrence in SurA-binding peptides has been reproduced for the sake of comparison [39]. For Small Tims, dark grey bars represent amino acids with a statistically significant association with bound/non-bound peptides ($p < 0.05$). For SurA, bars represent the relative occurrence in high (black bars), low (grey bars) and no (white bars) affinity binders.

(b) Proportion of bound (black) and non-bound (grey) peptides in peptide groups divided according to their amino acid content, hydrophobicity or net charge. The number of peptides in each group is indicated.

(c) Average hydrophobicity and average net charge of bound (black circles) and non-bound (grey circles) peptides as a function of the number of aromatic residues in the peptides.

Figure 6 SurA function in mitochondria

SurA and TIM10 share substrate binding specificity

- (a) Schematic representation of the SurA constructs used in this study.
- (b) Import of purified His-Tim10, purified intermembrane space-targeted SurA^{N_{Ct}} (pSurA^{N_{Ct}}) and radiolabelled SurA^{N_{Ct}} into wild-type mitochondria. After import, mitochondria were left intact (T), subject to osmotic swelling to remove the soluble IMS (MP), or subject to osmotic swelling in the presence of proteinase K, to remove soluble and peripherally-associated membrane proteins of the IMS (MP+PK). * Full-length pSurA^{N_{Ct}}, ** Mature SurA^{N_{Ct}}.
- (c) The yeast strain *P_{MET3}TIM10* supplemented with empty vector, Tim10 or intermembrane space-targeted pSurA^{N_{Ct}} were grown in medium lacking methionine. Serial ten-fold dilutions of these cultures were dropped onto plates of synthetic complete medium with (SC-U) or without (SC-M-U) methionine, and grown for 3 days at 30°C.
- (d) Import of Tim10, pSurA^{N_{Ct}} or mock import was carried out on Tim9^{ts} mitochondria, and unimported precursor was removed by treatment with trypsin then SBTI. Radiolabelled AAC was then imported, and half of each sample was treated with 25 µg/ml proteinase K (Lo) and the other half with 200 µg/ml proteinase K (Hi). Samples were loaded on SDS-PAGE and visualized by digital autoradiography. The arrow denotes the position of intact AAC and approximate molecular weights are indicated on the left hand side in kDa.

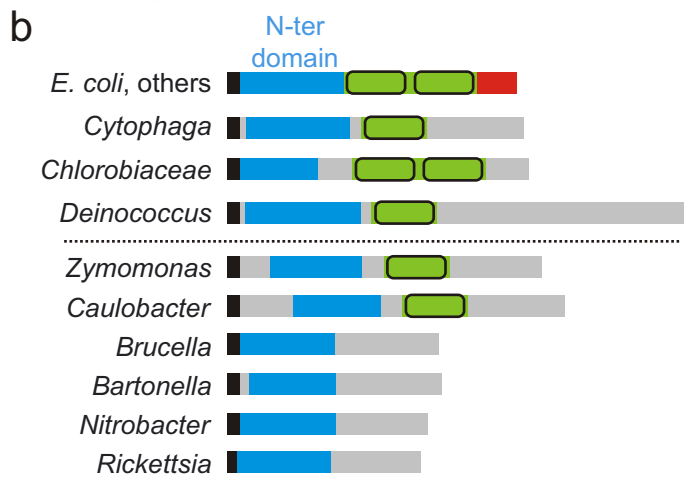
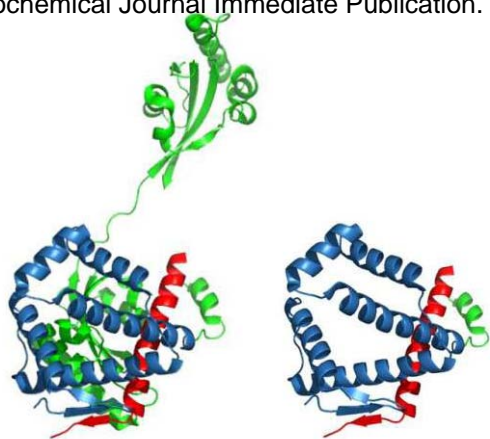


Figure 1

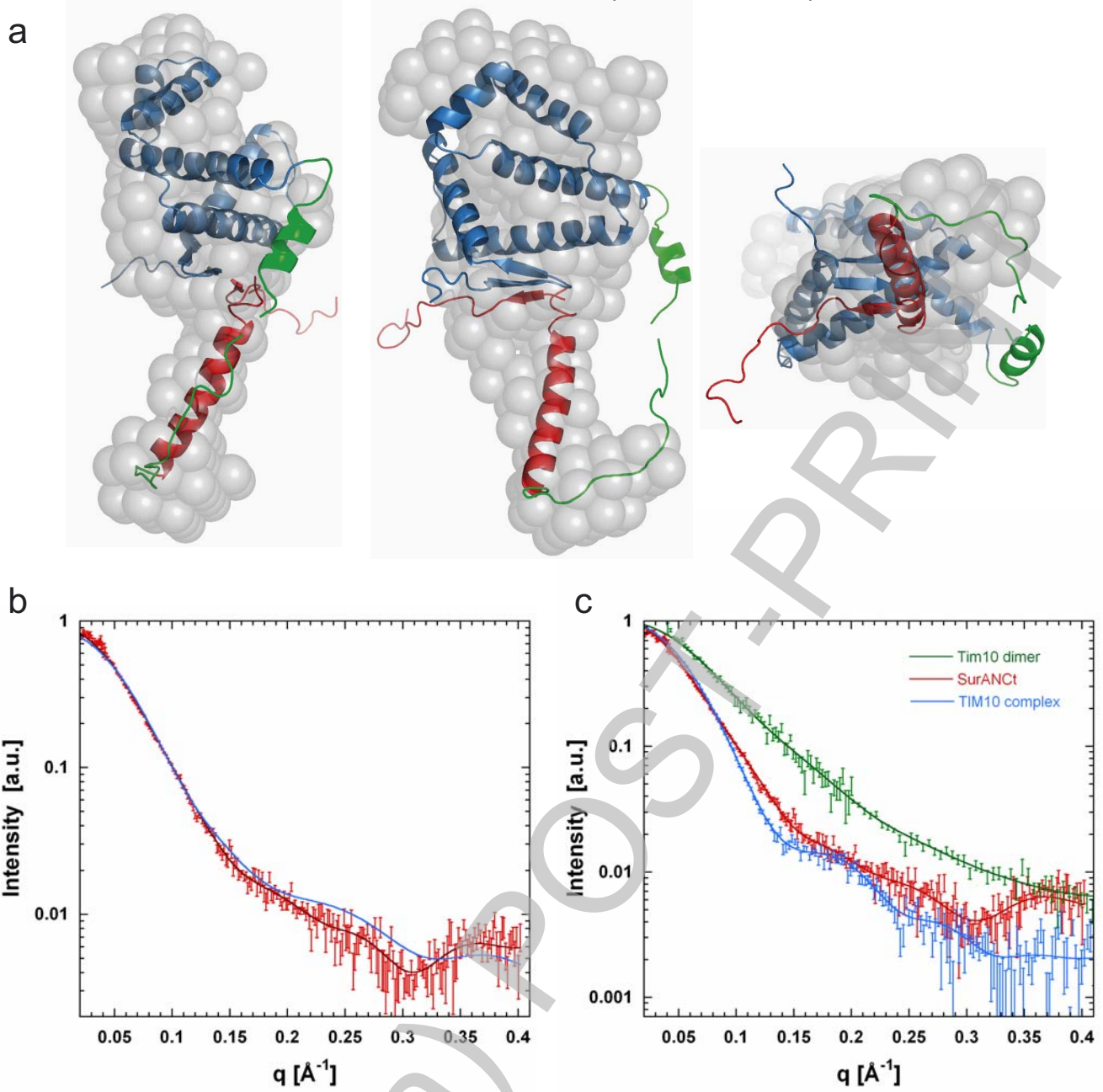


Figure 2

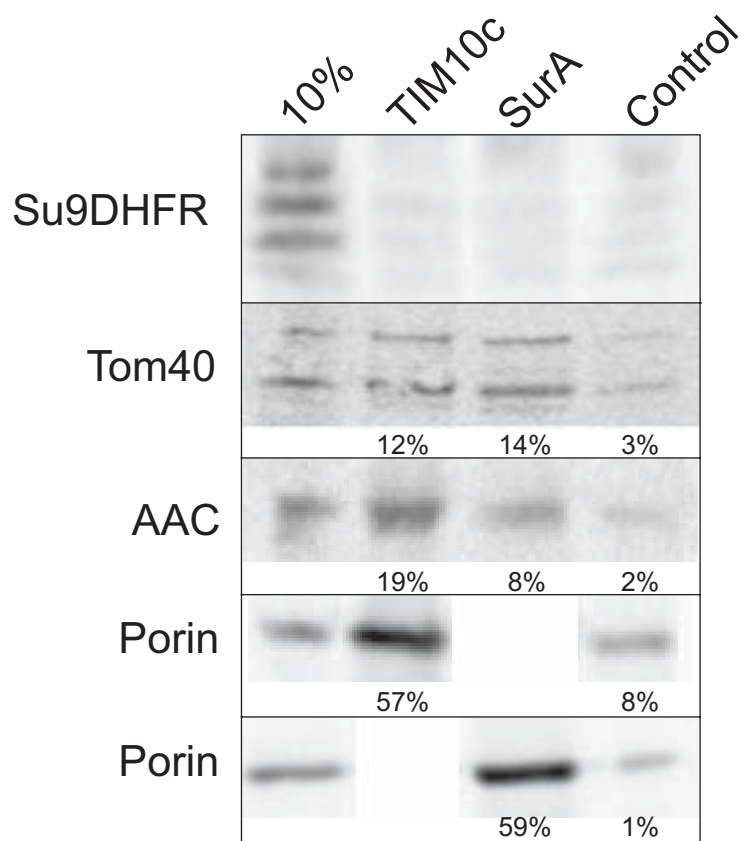


Figure 3

Stage 2(a) POST-PRINT

Tim22

AAC

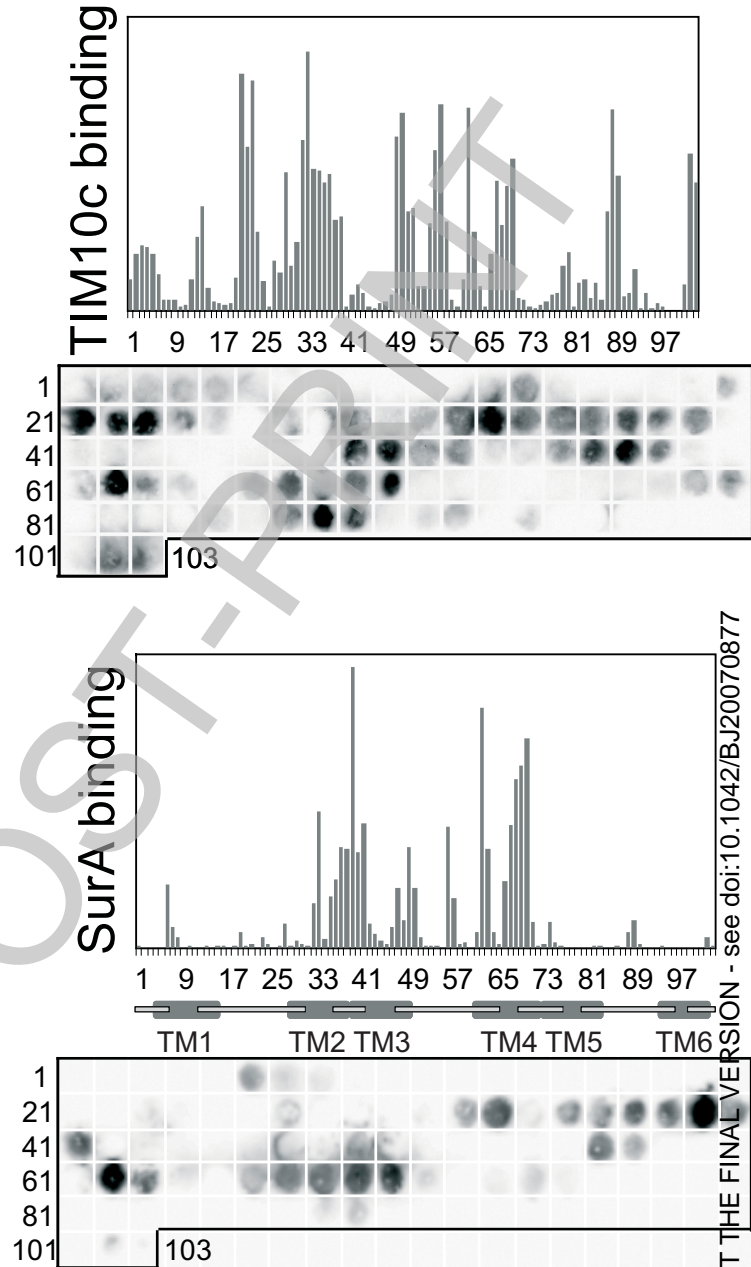
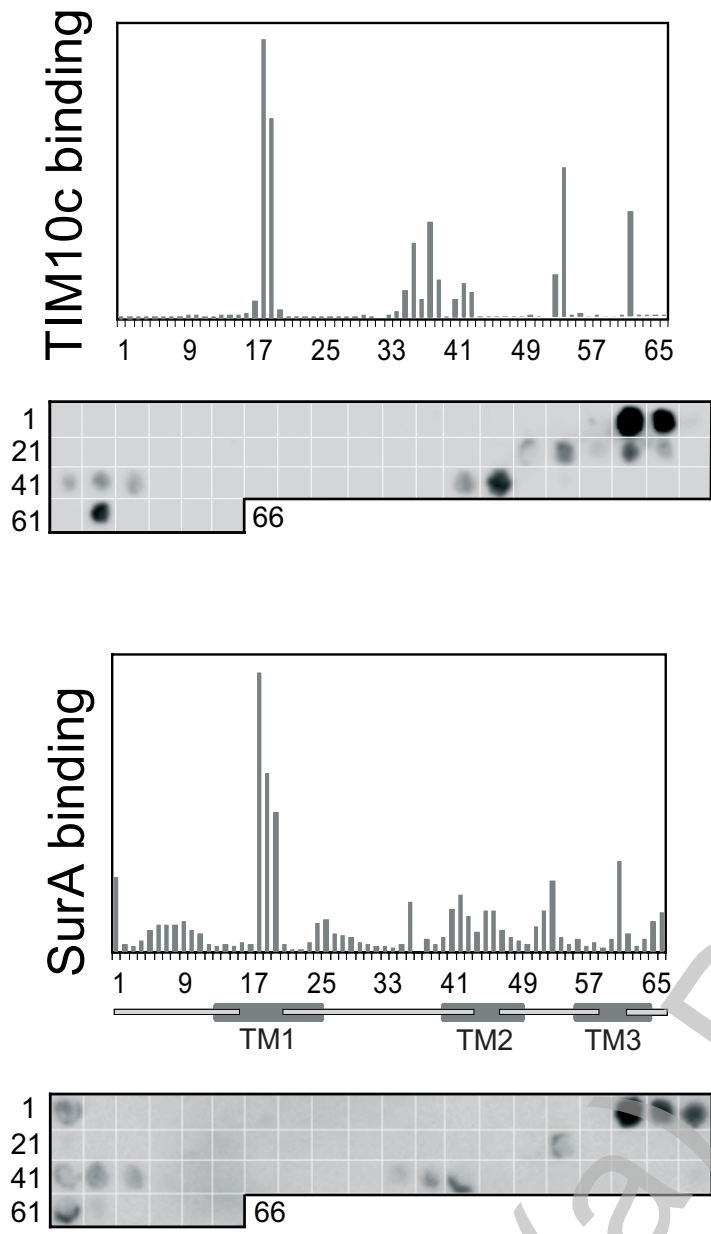


Figure 4

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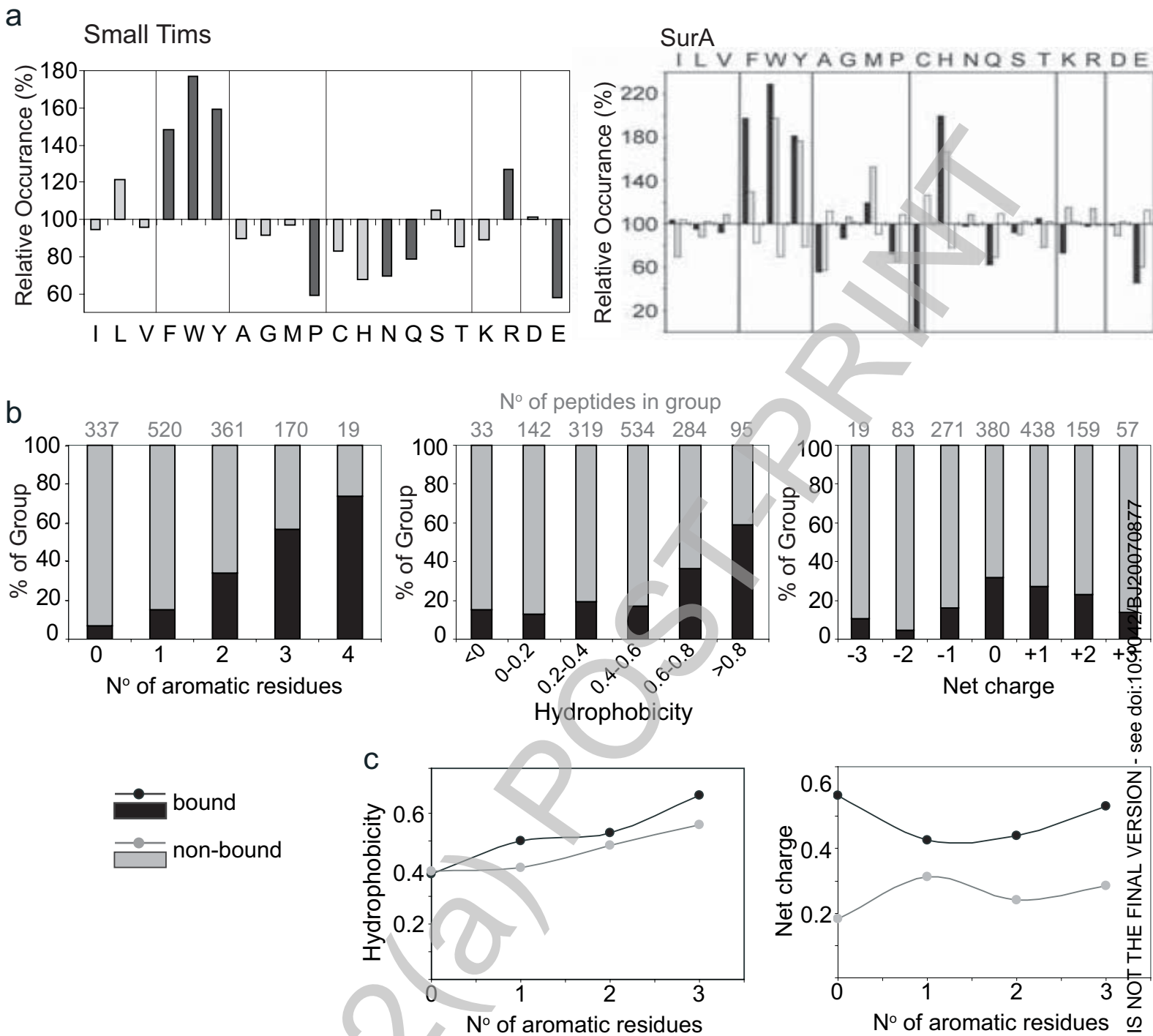


Figure 5

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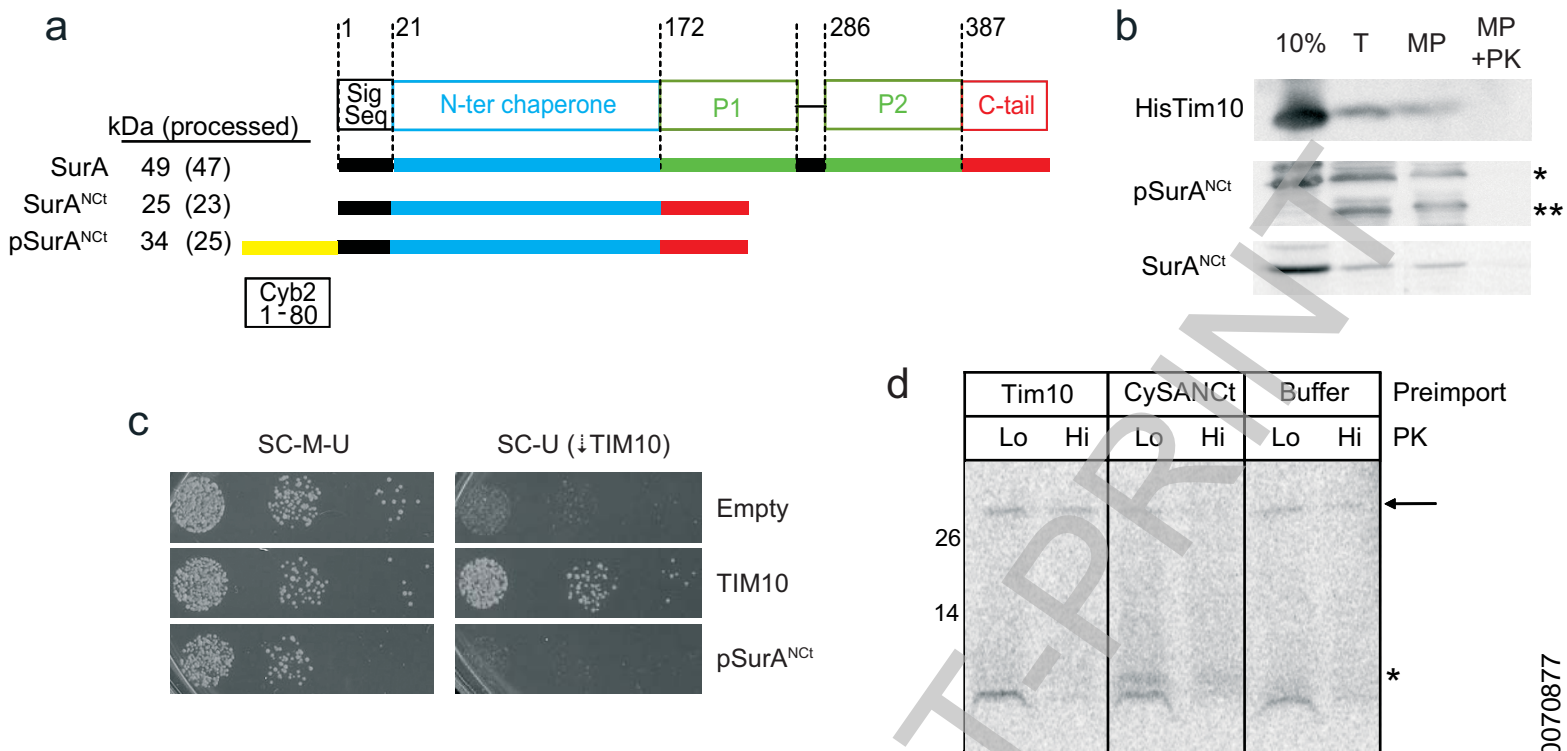


Figure 6