The Translocase of the Outer Membrane of Mitochondria (TOM Complex): Recognition of Mitochondrial Targeting Signals

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CONTENTS

1. INTRODUCTION

1.1. Origin, structure, and function of mitochondria

Eukaryotic cells are subdivided into various membrane-bounded compartments called cell organelles. The endoplasmic reticulum, the Golgi apparatus, lysosomes and peroxisomes possess one boundary membrane. In contrast to these organelles, mitochondria and chloroplasts are bordered by two membranes. Based on structural/functional similarities it was suggested that mitochondria are derived from bacteria which were incorporated into eukaryotic cells by a process called endosymbiosis (Margulis, 1981; Whatley, 1981). During evolution, mitochondria lost most of their genome. Today the vast majority of the mitochondrial proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes and thus have to be imported into mitochondria from the cytosol (Lang *et al.,* 1999). Mitochondrial proteins represent about 15-20% of all cellular proteins (Pfanner and Geissler, 2001).

Mitochondria have a complex structure. These organelles contain four subcompartments: the outer and inner membranes, and two aqueous compartments, the intermembrane space (IMS), and the matrix. The inner membrane, in comparison to the outer membrane, has a much larger surface. It can be subdivided into the inner boundary membrane and the cristae, which form invaginations (Palade, 1952; Frey and Mannella, 2000).

Mitochondria are the site of oxidative phosphorylation, as the complexes of the respiratory chain reside in the inner membrane. Mitochondria also house the citric acid (Krebs) cycle components in the matrix and are involved in important steps of the urea cycle, heme biosynthesis, fatty-acid metabolism, biosynthesis of phospholipids, amino acids, and nucleotides. The mitochondria are also involved in the synthesis of many coenzymes (Saraste, 1999; Scheffler, 2001). During the last years it was shown that mitochondria play an important role in apoptosis (programmed cell death), iron/sulfur cluster assembly, cancer, ageing, and signal transduction (Han *et al.,* 1998; Kim *et al.,* 2001; Martinou and Green, 2001; Voisine *et al.,* 2001; Zamzami and Kroemer, 2001).

Mitochondria are dynamic structures that are motile within the cells and undergo frequent changes in number and morphology, dividing and fusing continuously (Reichert and Neupert, 2002). These dynamic processes are enough to ensure an appropriate distribution of mitochondria during cell division, and adequate provision of ATP to those cytoplasmic regions where the energy consumption is particularly high (Yoon and McNiven, 2001). Mitochondria cannot be generated *de novo* by cells, as new mitochondria

form by division of pre-existing mitochondria. Growth occurs by insertion of newly synthesised constituents during the interphase period of the cell cycle.

1.2. Preprotein import into mitochondria

Newly synthesized mitochondrial preproteins contain specific targeting signals and are usually bound by factors which maintain the preproteins in a translocation-competent conformation. These are chaperones of the Hsp70 (Heat shock protein 70) family as well as specific factors like MSF (Mitochondrial import Stimulation Factor) that presumably recognize mitochondrial targeting signals (Murakami *et al.,* 1988; Komiya *et al.,* 1996; Mihara *et al.,* 1996). Recently, it was shown that the chaperone Hsp90, which has been thought to act largely on signal transducing proteins, in cooperation with Hsp70, mediates in mammals the targeting of a subset of mitochondrial preproteins (Young *et al.,* 2003).

Most mitochondrial preproteins are imported post-translationally (Neupert, 1997); however, translationally active ribosomes loaded with mRNA molecules encoding mitochondrial precursor proteins have been observed to accumulate on the surface of yeast mitochondria. Several recent observations support the idea that co-translational process is involved in the mitochondrial import of at least some proteins. It was proposed that mRNA localization to the vicinity of mitochondria plays a critical role in organelle biogenesis (Marc *et al.,* 2002; Margeot *et al.,* 2002)

The translocase of the outer mitochondrial membrane (TOM) mediates the entry of probably all nuclear encoded mitochondrial proteins into mitochondria. The TOM complex functions as a receptor for mitochondrial proteins and provides a protein conducting channel, through which mitochondrial proteins are threaded in an unfolded conformation (Eilers *et al.,* 1986). After crossing the outer membrane through the general import pore (GIP) of the TOM complex (discussed in detail later), imported preproteins are directed to one of two translocases of the inner membrane, the TIM complexes (Fig. 1).

All presequence-carrying preproteins are directed to the TIM23 complex which consists of the essential integral membrane proteins, Tim17, Tim23 and Tim50. These proteins associate with the membrane-bound Tim44 and the matrix heat shock protein mtHsp70 (Ryan *et al.,* 1993, Blom *et al.,* 1995; Yamamoto *et al.,* 2002). Both Tim17 and Tim23 have four putative membrane spanning domains and are partner proteins in a 90 kDa complex (Emtage and Jensen, 1993; Kübrich *et al.,* 1994).

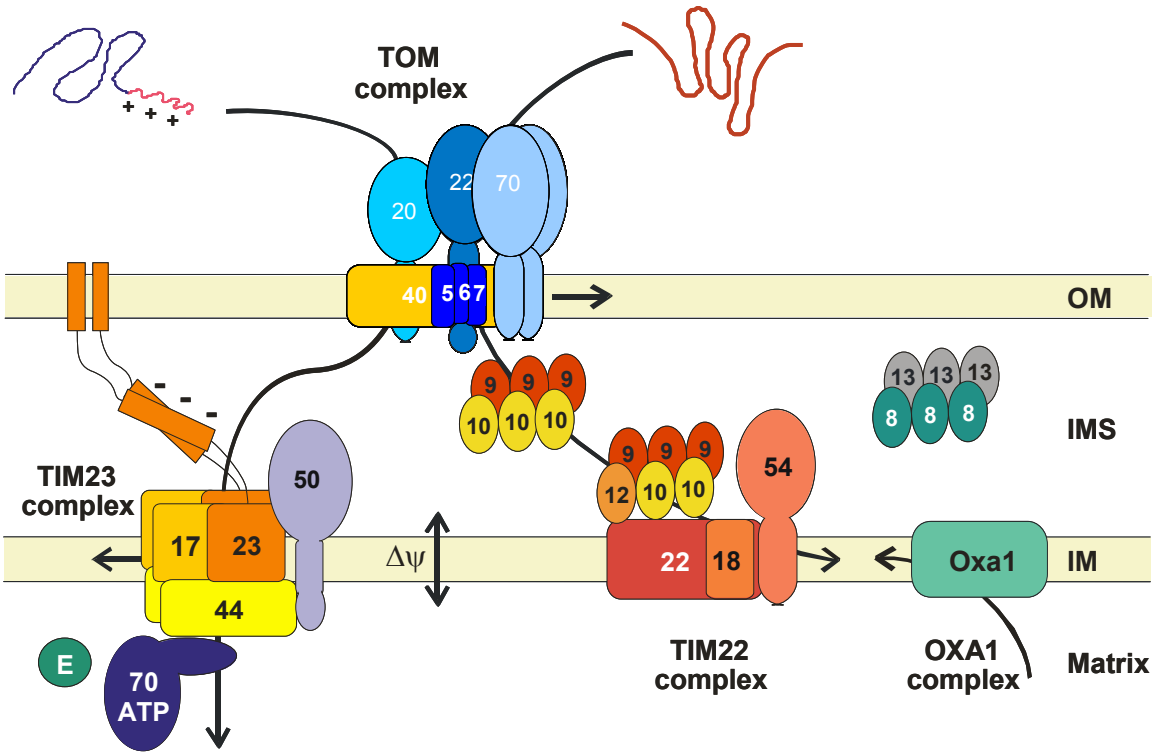

Fig. 1. The general import pathway into mitochondria. Preproteins first bind to specialized import receptors of the TOM complex at the outer membrane and then are transferred to the general insertion pore. For further translocation, the TOM complex cooperates with the TIM23 and TIM22 complexes in the inner membrane. The OXA1 complex in the inner membrane mediates insertion of precursors from the matrix space into the inner membrane (adapted from Bauer *et al.,* 2000).

Tim23 contains a negatively charged domain in the intermembrane space that recognizes precursors taking the general import route (Bauer *et al.,* 1996). It is proposed that its amino terminus extends into the outer membrane and links both mitochondrial membranes (Donzeau *et al.,* 2000). Purified Tim23, reconstituted into liposomes seems to form a voltage-sensitive high-conductance channel (Truscott *et al.,* 2001). Tim23 has been proposed to form a dimer in the absence of a membrane potential such that the import channel is closed (Bauer *et al.,* 1996). Precursor binding to the intermembrane space domain triggers dimer dissociation, allowing the precursor to pass through the import channel. Tim50 is an integral membrane protein, exposing the C-terminal domain to the intermembrane space and interacting with the N-terminal intermembrane space domain of Tim23. Tim50 is proposed to facilitate transfer of the translocating protein from the TOM complex to the TIM23 complex (Geissler *et al.,* 2002; Yamamoto *et al.,* 2002; Mokranjac *et al.,* 2003). Protein translocation across the inner membrane to the matrix requires two driving forces: a membrane potential across the inner membrane and an ATP-dependent import motor, which consists of mtHsp70, Tim44 and the co-chaperone Mge1. All three

components of the import motor are essential for viability. Two models have been proposed to explain the role of mtHps70 in protein import: (1) the Brownian ratchet in which random motion is translated into vectorial motion, and (2) a "power stroke", which is exerted by a component of the import machinery (Neupert and Brunner, 2002).

In the Brownian ratchet model, mtHsp70 represents the arresting component of a ratchet, which allows forward, but not backward, movement of the polypeptide chain; spontaneous Brownian forward movement can be transduced into vectorial transport by cycles of mtHsp70 binding (Ungermann *et al.,* 1996; Chauwin *et al.,* 1998; Gaume *et al.,* 1998). Further support for this model was obtained by two different approaches: preproteins containing stretches of glutamic acid or glycine repeats, (polyE and polyG, respectively) in front of folded domains were imported into mitochondria. This occurred although Hsp70 cannot pull on these stretches to unfold the folded domains, since it does not bind to polyE and polyG. Secondly, preproteins containing titin immunoglobulin-like domains were imported into mitochondria, despite the fact that forces of >200 pN are required to mechanically unfold these domains. Since known molecular motors generate forces of approximately 5 pN, Hsp70 could not promote unfolding of the immunoglobulin -like domains by mechanical pulling (Okamoto *et al.,* 2002).

The power-stroke model proposes that mtHsp70 undergoes a conformational change and pulls on the polypeptide chain. Multiple cycles of mtHsp70 binding would lead to regular stepwise translocation (Horst *et al.,* 1997; Krimmer *et al.,* 2001).

After import into the matrix, the targeting signals of the imported proteins are cleaved off by mitochondrial processing peptidase (MPP). In the case of some preproteins that are destined for the intermembrane space (IMS) two cleavages take place. The first cleavage is by MPP; in a second processing event a sequence encoding a sorting signal for the IMS is cleaved by the Imp1 and/or Imp2 proteases at the outer face of the inner membrane (e.g. cytochrome b_2, Cox2) (Nunnari *et al.,* 1993). Complete removal of the presequences and folding to the native state are essential prerequisites for obtaining the functional conformation of imported proteins. Two major chaperone classes in the mitochondrial matrix, Hsp70 and Hsp60 operate in the folding reactions of the imported proteins. MtHsp70 interacts with the co-chaperones Mdj1 and Mge1. Some proteins however do not need mtHsp70 to reach their native conformation (Schilke *et al.,* 1996). For a subset of mitochondrial proteins, folding mediated by Hsp60 is essential for the acquisition of the native conformation. Members of the third family of mitochondrial chaperones, the Clp or Hsp100 proteins, perform important roles during the later stages of the life cycle of some proteins (Voos and Röttgers, 2002).

Whereas the TIM23 complex is preferentially used by presequence-carrying hydrophilic matrix proteins and inner membrane proteins with a limited number of transmembrane segments, other inner membrane proteins, in particular those containing multiple membrane-spanning domains are targeted to the TIM22 complex. This preprotein translocase inserts them into the inner membrane in a membrane potential-dependent manner (Sirrenberg *et al.,* 1996; Bömer *et al.,* 1997; Kerscher *et al.,* 1997; Koehler *et al.,* 2000). Only two membrane integrated components of this complex are known in *N. crassa*, Tim22 and Tim54; a third membrane protein, Tim18, was identified in *S. cerevisiae*, but has not been detected in the *N. crassa* genome. Tim22, an essential inner membrane protein, is structurally related to the Tim23 and Tim17. These observations suggest that these translocases might have evolved by gene-duplication events (Bauer *et al.,* 2000). Tim54 contains one or perhaps two predicted membrane-spanning segments and is required for the maintenance of Tim22 (Kerscher *et al.,* 1997). The TIM22 complex interacts with three small, structurally related proteins of the intermembrane space, Tim9, Tim10 and Tim12, which are also required for carrier translocation (Sirrenberg *et al.,* 1996; Koehler *et al.,* 1998). Tim9, Tim10 and Tim12 are organized probably in two types of hetero-oligomeric 70 kDa complexes. The TIM9-10 complex is reported to contain three molecules of Tim9 and three molecules Tim10. In contrast, the TIM9-10-12 complex probably consists of three molecules of Tim9, two molecules of Tim10 and one molecule of Tim12. The TIM9-10-12 complex is loosely associated with the membrane-integrated components of the TIM22 complex, whereas the TIM9-10 complex is largely soluble in the intermembrane space. Tim18, Tim22 and Tim54 together with small Tim proteins of the intermembrane space form a complex of 300 kDa. The exact function of Tim18 and Tim54 is still unclear. *S. cerevisiae* encodes two proteins, Tim8 and Tim13 that are structurally related to Tim9, Tim10 and Tim12. Both proteins are localized in the intermembrane space and are organized in hetero-oligomeric 70 kDa complexes. They were proposed to be involved in the import of subset of mitochondrial inner membrane proteins such as Tim23 (Paschen *et al.,* 2000; Curran *et al.,* 2002).

A subset of inner membrane proteins (including proteins encoded by nuclear and mitochondrial DNA) are sorted by way of insertion from the mitochondrial matrix into the inner membrane. The protein translocase involved in their pathway is the OXA1 complex (Stuart and Neupert, 1996). Oxa1p is a member of the highly conserved Oxa1p/YidC/Alb3 protein family found throughout prokaryotes and eukaryotes (Bauer *et al.,* 1994; Bonnefoy *et al.,* 1994). Examples of proteins that use the OXA1 complex for their membrane insertion include the mitochondrially encoded subunit 2 of the cytochrome oxidase complex, Cox2p

which spans the inner membrane twice and Oxa1p itself, a nuclear encoded polytopic protein that spans the membrane five times (He and Fox, 1997; Hell *et al.,*1997).

1.3. Mitochondrial targeting signals

Targeting signals are defined as sequences in preproteins that are both necessary and sufficient to direct proteins to mitochondria (Neupert, 1997). The classical mitochondrial targeting signal is an amino-terminal cleavable presequence, which functions as a matrix-targeting signal. When attached to non-mitochondrial passenger proteins, presequences can specifically direct the passenger across both mitochondrial membranes into the matrix (Hurt *et al.,* 1984; Horwich *et al.,* 1985). Presequences comprise ca. 20-60 amino acid residues. These sequences are not conserved between different proteins and only weakly between homologus proteins in different species. A common element is the abundant occurrence of positively charged, hydroxylated and hydrophobic amino acid residues and the absence (with few exceptions) of negatively charged residues. The presequences have the potential to form an amphipathic α-helix with a positively-charged face on one side and a hydrophobic surface on the other. This helical structure appears to exist however only in a membranous or in membrane-like environment; in aqueous environments they do not seem to be dominant (Roise *et al.,* 1988). The amphipathic structure of the presequences is thought to be important for their specific recognition by the protein import machinery (Abe *et al.,* 2000). Whereas the presequences of most matrix proteins are cleaved off upon import by the mitochondrial processing peptidase (MPP), several matrix proteins, such as rhodanese, 3-oxo-acyl-CoA-thiolase and chaperonin 10 (Hsp10) are synthesised with a non-cleavable N-terminal targeting signal which has characteristics very similar to those of the cleaved signals (Jarvis *et al.,* 1995; Waltner *et al.,* 1995; Hammen *et al.,* 1996). One matrix protein, the DNA helicase Hmi1, so far has been found to contain a presequence-like targeting signal at its carboxy terminus (Lee *et al.,* 1999).

Signals resembling presequences are found in several preproteins of the outer membrane, the intermembrane space, and the inner membrane. In these cases, the positively charged sequences are followed by hydrophobic sorting signals that lead to the specific arrest of the preproteins in the outer or inner membranes (Glick *et al.,* 1992; McBride *et al.,* 1992; Hahne *et al.,* 1994; Gärtner *et al.,* 1995). For example, the outer membrane protein Tom70 contains at its N-terminal a positively charged stretch followed by a hydrophobic segment. These two structural elements contain the information for the targeting and insertion into the outer membrane of Tom70 (McBride *et al.,* 1992).

A bipartite presequence is used to sort some proteins to the inner membrane (e.g.

cytochrome c_1) or the intermembrane space (e.g. cytochrome b_2). In this case, a positively charged matrix-targeting sequence is followed by a sorting sequence including a hydrophobic stretch preceded by a few positively charged residues. It was proposed that these sorting sequences act as stop-transfer signals arresting passage of the precursor through the inner membrane. These sorting sequences are cleaved off at the outer surface of the inner membrane by the heterodimeric inner membrane peptidase (Imp1-Imp2) (Glick *et al.,* 1992). It has not been resolved so far whether these proteins are first completely or partially imported into the matrix and then redirected into the inner membrane, thereby resembling prokaryotic protein export ("conservative sorting") (Hartl *et al.,* 1987; Fölsch *et al.,* 1996).

Some inner membrane proteins contain internal targeting signals that appear to consist of a transmembrane segment and a positively charged segment directly after it. This internal signal is thought to form a hairpin-loop structure in the inner membrane (Fölsch *et al.,* 1996). Proteins of the metabolite carrier family of the inner membrane do not contain a cleavable presequence, but have multiple signals distributed throughout the entire length of the preprotein (Pfanner *et al.,* 1987; Smagula and Douglas, 1988a, Endres *et al.,* 1999). Other membrane proteins, such as components of the inner membrane translocases (Tim17, Tim23 and Tim22) also contain several targeting and sorting signals, including hydrophobic segments and positively charged loops (Kaldi *et al.* 1998; Davis *et al.,* 2000; Paschen and Neupert, 2001).

1.4. The TOM complex

The outer membrane translocase, the TOM complex, is a multisubunit complex of ca. 450 kDa composed of seven subunits (the so-called TOM holo complex): Tom70, Tom40, Tom22, Tom20, Tom7, Tom6, Tom5 (Neupert, 1997; Pfanner *et al.,* 2001) (Fig.2). The TOM complex mediates the translocation across and insertion into the outer membrane of virtually all nuclear encoded mitochondrial preproteins. Hence, it should be able to recognize and decode all types of mitochondrial targeting signals. Preproteins are recognized on the mitochondrial surface by the receptor subunits of the TOM complex, Tom20, Tom22, and Tom70 (Söllner *et al.,* 1989; Hines *et al.,* 1990; Moczko *et al.,* 1992; Hines and Schatz, 1993; Kiebler *et al.,* 1993). Subsequently, preproteins are transferred into the protein conducting channel of the TOM complex, also known as the general import/insertion pore (GIP), and translocated through the outer membrane into the intermembrane space. The GIP is part of the "TOM core complex", which is composed of Tom40, Tom22, Tom7, Tom6, and Tom5, but does not contain the receptor subunits Tom20

and Tom70 (Ahting *et al.,* 1999).

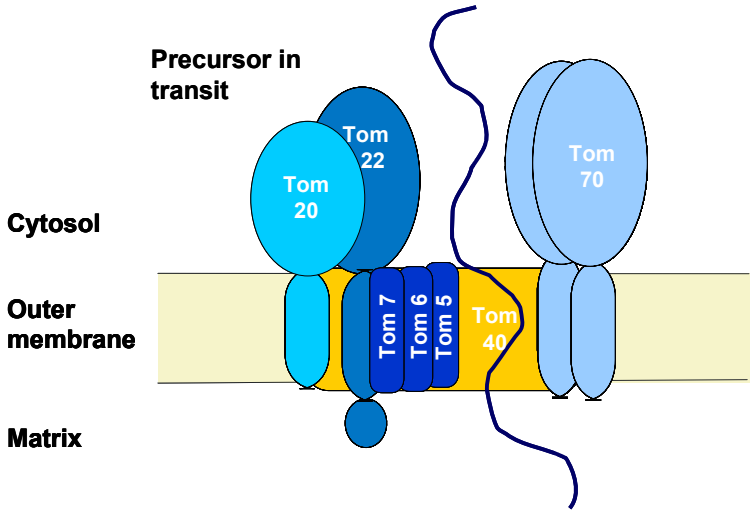

Fig. 2. Translocase of the outer mitochondrial membrane (TOM complex). The TOM complex contains the initial receptors Tom20 and Tom70. These two receptors are loosely attached to the rest of the complex (the TOM core complex); they are coisolated with the other Tom components when mild detergents are used to solubilize mitochondria, but not under more rigorous conditions. The TOM core complex forms the protein-conducting pore and contains Tom5, Tom6, Tom7, Tom22 and Tom40. The names of the Tom subunits reflect their molecular weights (adapted from Rapaport, 2002).

The two receptor proteins Tom20 and Tom70 show different, but partially overlapping specificities for preproteins (Lithgow *et al.,* 1995). The observation that single deletion of either receptor can be tolerated suggests that the receptors can partly substitute for each other. Double deletion is lethal (Ramage *et al.,* 1993). Tom70 contains seven predicted tetratricopeptide repeat (TPR) motifs while Tom20 contains one such domain. This motif may have a role in protein-protein interaction (Haucke *et al.,* 1996, Young *et al.,* 2003). Both Tom70 and Tom20 contain an amino-terminal membrane anchor and a hydrophilic C-terminal cytosolic domain of 65 kDa and 17 kDa, respectively (Söllner *et al.,* 1989). Tom20 recognizes mainly proteins that carry presequences, but recently some proteins were found that bind to Tom20 although they lack a mitochondrial presequence, like the outer membrane proteins porin (Schleiff *et al.,* 1997), Tom40 (Rapaport and Neupert, 1999), the intermembrane-space protein cytochrome *c* heme lyase (Diekert *et al.,* 1999) as well as the inner membrane protein Tim22. Recently the molecular basis for the presequence binding to Tom20 was analysed by NMR analysis (Abe *et al.,* 2000). The presequence binding pocket of Tom20 is formed by three α-helices that create a hydrophobic patch lacking charged and hydrophilic amino acid residues. This patch

interacts with the hydrophobic face of the amphipathic α-helix of the presequence, whereas the positively charged and hydrophilic amino acid residues on the alternate face of the α-helix are exposed towards the aqueous solvent. In addition, interactions of the presequence with the cytosolic domain of the Tom20 are probably mediated by ionic interactions, which might explain the essential nature of the positively charged and hydroxylated amino acids of the N-terminal presequences.

Tom70 binds preferentially to hydrophobic preproteins that contain internal targeting information. Tom70 has a tendency to form dimers, and the membrane anchor is responsible for, or at least contributes, to dimerization (Söllner *et al.,* 1992; Millar and Shore, 1994). Two other receptor components were identified in *S. cerevisiae*: Tom71 and Tom37. Despite the fact that Tom71 is closely related to Tom70 (53% sequence identity, 70% similarity), the two receptors do not perform identical functions: the import of Tom70-dependent preproteins is minimally affected by the deletion of Tom71, irrespective of the presence or absence of the Tom70 receptor (Schlossmann *et al.,* 1996). Tom37 by itself is a non-essential membrane protein with two putative transmembrane domains, but double mutants of tom37Δ with tom70Δ or with tom20Δ are lethal (Gratzer *et al.,* 1995). The receptor components were suggested to form two dynamically interacting subcomplexes: a heterodimer composed of Tom70 and Tom37 and a second one composed of Tom20 and Tom22 (Gratzer *et al.,* 1995, Mayer *et al.,* 1995b).

Both receptor complexes transfer the bound preproteins to GIP. In this context Tom22 plays an important role. First, Tom22 interacts with the presequences and serves as an additional or accessory import receptor. Second, Tom22 is an integral part of the general insertion pore (van Wilpe *et al.,* 1999). Tom22 mediates the interaction of the Tom20 with the GIP, and it appears to interact with the hydrophilic side of presequences (Brix *et al.,* 1997). Tom22, together with Tom5, mediates the insertion of the polypeptide chain into the GIP. Tom22 extends an N-terminal domain of 85 amino acid residues in the cytosol, has a single transmembrane segment, and has a smaller C-terminal domain (45 residues) facing the intermembrane space. The cytosolic domain of Tom22 is characterized by an abundance of negative charges.

The preprotein conducting channel of the GIP is probably formed by several Tom40 molecules, the only essential Tom protein in yeast. Tom40 spans the membrane presumably in a porin-like manner with several β-strands that form a β-barrel with a pore of ca. 2 nm in width (Hill *et al.,* 1998; Künkele *et al.,* 1998). Recombinant *S. cerevisiae* Tom40 or native Tom40 from *N. crassa* were reconstituted into liposomes. They form a cation-selective high-conductance channel to which mitochondrial targeting sequences added to the *cis* side

of the membrane specifically bind (Hill *et al.,* 1998; Ahting *et al.,* 2001). It was also proposed that Tom40p simultaneously associates efficiently with itself and preferentially recognizes the targeting sequence of mitochondrial precursor protein (Rapaport *et al.,* 1997; Rapaport *et al.,* 1998b; Gordon *et al.,* 2001).

The isolated native Tom40 of *N. crassa* is organized in a high molecular mass complex of approximately 350 kDa (Ahting *et al.,* 2001). Electron microscopy of purified Tom40 revealed particles primarily with one center of stain accumulation. They presumably represent an open pore with a diameter of approximately 2.5 nm, similar to the pores found in the TOM complex (Künkele *et al.,* 1998; Ahting *et al.,* 2001).

The additional TOM core subunits Tom22, Tom7, Tom6, and Tom5 are necessary for the stability and the dynamic regulation of the complex. The functions of the small Tom proteins, Tom7, Tom6, and Tom5 of the *S. cerevisiae* are only partially understood. Tom5 is believed to exert a receptor-like function by taking over preproteins from the Tom22 receptor, but it is also needed for the subsequent insertion of polypeptide chains into the translocation pore (Dietmeier *et al.,* 1997). Tom6 and Tom7 seem to be involved in the regulation of the assembly and disassembly of receptor proteins with the GIP (Hölinger *et al.,* 1996; Dekker *et al.,* 1998). Recently it has been shown that *N. crassa* Tom6 and Tom7 are in the vicinity of Tom40, while Tom6 forms probably the link between Tom40 and Tom22 (Dembowski *et al.,* 2001).

For the translocation of preproteins across the outer membrane, neither the membrane potential nor ATP is necessary. For matrix-targeted preproteins it was proposed that the TOM complex provides binding sites on each side of the outer membrane. The cytosolic domains of Tom20, Tom22, and Tom5 form the *cis* site of the TOM complex, which binds the presequence reversibly in a salt sensitive manner (Mayer *et al.,* 1995c; Bollinger *et al.,* 1995). Tom40 seems to be largely responsible for the formation of the *trans* site, which is localized at the inner face of the membrane (Rapaport *et al.,* 1997). *Trans* site binding occurs with much higher affinity than *cis* site binding (Mayer *et al.,* 1995; Rapaport *et al.,* 1998b). It seems likely that the translocation across the outer membrane is driven by the sequential interaction of the presequences with different modules of the TOM complex that bind the preproteins with increasing affinity.

1.5. The BCS1 protein

The ubiquinol-cytochrome *c* oxidoreductase (cytochrome bc_1 complex) of the respiratory chain is composed of three catalytic subunits: the mitochondrially encoded cytochrome *b*, the nuclear encoded cytochrome c_1 and Rieske FeS protein. These proteins,

together with a series of other non-catalytic subunits assemble to form an enzymatically active complex. In *S. cerevisiae* these non-catalytic subunits are Core1, Core2, Qcr6p, Qcr7p, Qcr8p Qcr9p and Qcr10p (Tzagoloff, 1995). Cytochrome *b* initially forms a subcomplex with Qcr7p and Qcr8p, which subsequently joins with the Core1 and Core2 proteins. Cytochrome c_1, on the other hand, is proposed to form another subcomplex with Qcr6p and Qcr9p (Grivell, 1989). The cytochrome *b* and the cytochrome c_1 complexes subsequently unite to form the "cytochrome bc_1 precomplex", prior to the assembly of the Rieske FeS protein and presumably the non-essential subunit, Qcr10p (Xia *et al.,* 1997; Zhang *et al.,* 1998) (Fig. 3).

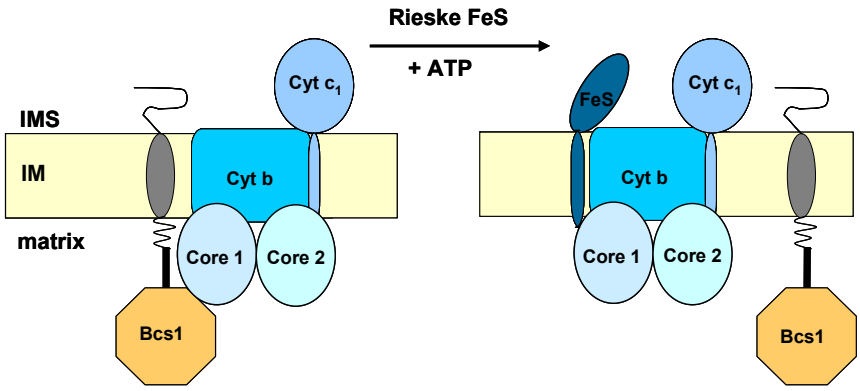

Fig. 3. Model of ATP-dependent interaction of BCS1 with the cytochrome bc_1 pre-supracomplex. BCS1 interacts in an ATP-dependent manner with the cytochrome bc_1 precomplex; it appears to maintain it in a state competent for the subsequent assembly of the Rieske FeS protein. IM, inner membrane; IMS, intermembrane space; FeS, Rieske FeS protein; Cyt b, cytochrome *b*; Cyt c_1, cytochrome c_1 (adapted from Cruciat *et al.,* 1999).

BCS1 is a mitochondrial inner membrane protein and a member of the highly conserved ATPases, the AAA protein family (ATPases associated with different cellular activities). Members of this diverse family are united by their conserved AAA sequence motif, which encompasses an ATP-binding site comprising Walker A and B boxes. AAA family members are involved in a variety of cellular processes, including vesicle-mediated transport, proteolytic degradation, and cell cycle regulation (Beyer, 1997; Patel and Latterich, 1998).

BCS1 was identified upon characterization of a yeast mutant deficient in respiratory activity. Deletion of the gene causes a deficiency in the assembly of cytochrome bc_1 complex; a precomplex is found that lacks the Rieske FeS protein.

BCS1 interacts in an ATP-dependent manner with the cytochrome bc_1 precomplex; it appears to maintain it in a state competent for the subsequent assembly of the Rieske FeS

protein. At the cytochrome bc_1 precomplex, BCS1 occupies a site subsequently taken by the Rieske FeS protein, suggesting that both proteins could share limited structural similarities (Cruciat *et al.,* 1999).

The BCS1 protein is anchored in the mitochondrial inner membrane via a single transmembrane domain (residues 45-68). A short N-terminal tail is exposed into the intermembrane space (residues 1-44), whilst the bulk of the protein is in the matrix (in a N_{out}-C_{in} orientation). The transmembrane anchor is followed by a short positively charged segment (residues 69-83) that is predicted to form an amphiphilic helical structure (Fig. 3). This segment contains a potential MPP processing site that is not cleaved when present in the context of an internal targeting sequence (Fölsch *et al.*, 1996). The N-terminal sequence of BCS1 does not display any resemblance to a mitochondrial targeting signal, in particular containing more negative than positive charges.

Import of BCS1 across with the inner mitochondrial membrane was studied in some detail by Fölsch *et al.*, 1996. It was proposed that during its translocation across the inner membrane, which is mediated by the TIM23 complex, a tight loop structure is formed in which the transmembrane domain contacts the hydrophobic site of the amphiphilic α-helix. However, the mechanism by which the TOM complex recognizes and translocates the BCS1 precursor remains to be elucidated.

1.6. Aims of the present study

The main goal of this study was to obtain new insights into the mechanism by which the TOM complex recognizes and decodes different types of mitochondrial targeting signals.

The possibility to isolate detergent solublilized TOM complex provided a tool to study whether precursor proteins are recognized by the TOM complex, in the absence of cytosolic chaperones and lipids in a bilayer structure.

Another aim of this study was to use BCS1 as model protein to understand what the signals are in a precursor with internal targeting and sorting information and how these signals are decoded by the mitochondrial TOM complex. Additional questions were which components of the TOM complex can mediate this recognition and in which conformation BCS1 interacts with the TOM complex upon translocation across the outer membrane.

2. MATERIAL AND METHODS

2.1. *Molecular biology methods*

2.1.1. Small and large scale isolation of plasmid DNA from *E.coli*

Small scale preparation of plasmid DNA was performed according to the alkaline lysis method (Birnboim and Doly, 1979). LB-medium (2 ml) containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated (ON, 37°C) under vigorous agitation conditions. Cells from 1.5 ml culture were harvested by centrifugation (7,500xg, 30 sec). The resulting cell pellet was resuspended in 300 µl buffer E1 (50 mM Tris-HCl, 10 mM EDTA-Na_2 x H_2O, 37% HCl, pH 8.0) containing 100 mg/ml RNase, and cell lysis was performed by adding 300 µl buffer E2 (0.2 M NaOH, 1% SDS). The samples were mixed by inverting the tubes 5 times and left for 5 min at RT. For neutralization, 300 µl buffer E3 (3.1 M KOAc, pH 5.5) was added, and the samples were mixed immediately by inverting the tubes 5 times. After centrifugation (35,000xg, 10 min), the supernatant (containing the plasmid DNA) was transferred to a new tube and the DNA was precipitated by adding 600 µl isopropanol (96%). The samples were pelleted via centrifugation, washed with 70% cold ethanol and, after drying at RT, resuspended in 30 µl H_20 and used for further analysis.

For large scale preparation of plasmid DNA (up to 0.5 mg) a "Jetstar" Kit (Genomed) was used. LB-medium (50 ml) supplemented with the appropriate antibiotic was inoculated with bacteria carrying the required plasmid and incubated (ON, 37°C) under vigorous agitation conditions. The bacteria were harvested by centrifugation (3,000xg, 10 min) and resuspended in 4 ml of buffer E1 and cells lysis was performed by adding 4 ml of buffer E2. The samples were mixed by inverting the tubes 5 times and left for 5 min at RT. After neutralization by adding 4 ml of buffer E3, samples were centrifuged (31,000xg, 10 min, at RT). The supernatant was applied to an anion-exchange column, previously equilibrated with 10 ml buffer E4 (0.15% v/v Triton X-100, 0.6 M NaCl, 100 mM NaOAc, pH 5.0). The column was washed twice with 10 ml buffer E5 (0.8 M NaCl, 100 mM NaOAc, pH 5.0) and the plasmid was eluted by adding 5 ml buffer E6 (1.25 M NaCl, 100 mM Tris-HCl, pH 8.5). The DNA was precipitated with isopropanol, sedimented (23,700xg, 30 min, 4°C), washed with 70% ethanol, dried at RT and resuspended in a small volume of H_2O.

2.1.2. Preparation of yeast DNA

Preparation of yeast DNA was performed as previously described (Rose *et al.,* 1990). YPD-medium (5 ml) was inoculated with *S. cerevisiae* cells and incubated (ON, 30°C) under shaking conditions. The cells were harvested by centrifugation, washed with H_2O, and resuspended in 200 µl buffer (2% Triton-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). A mix: Phenol/Chloroform/ Isoamyl alcohol (25:24:1) (200µl) and 0.3 g glass beads were added and the samples were vortexed for 2 min and centrifuged (35,000xg, 5 min). The supernatant (the aqueous phase) was transferred to a new tube and the DNA was precipitated by adding 2.5 vol. of 100% ethanol. The samples were kept for 10 min at – 20°C, centrifuged (35,000xg, 10 min), and washed with 70% ethanol. The pellets were dried at RT, resuspended in 40 µl H_2O and used for analysis.

2.1.3. Polymerase Chain Reaction (PCR)

The DNA sequences were amplified by Polymerase Chain Reaction (PCR), using thermostable DNA polymerase. PCR mix contains (total 100 µl): 1-2 U DNA polymerase (*Taq*-polymerase and/or *Pfu*-polymerase), 10 µl PCR-buffer (1% Triton X-100, 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.8), 2 µl dNTPs (10 mM stock), 50 pM primers and 200 ng plasmid DNA template or 1 µg genomic DNA template.

The following program was used:

1) 3 min, 94°C	nuclease inactivation and complete DNA denaturation;	
2) 20-30 cycles:	1 min, 94°C	DNA denaturation;
	1 min, 45-65°C	annealing of oligonucleotide primers;
	1-6 min, 72°C	new DNA synthesis (extension) (The duration of this step is determined by the length of the DNA fragment to be amplified) *Taq*-poymerase: 1 min/1kb *Pfu*-polymerase: 2.5 min/1kb;
3) 5-20 min, 72°C	completion of the last reaction.	

The amplified DNA fragments were analyzed by agarose gel electrophoresis.

2.1.4. Enzymatic manipulation of DNA

For analytical and preparative purposes plasmid DNA was digested with specific restriction endonuclease (up to 5 U of enzyme for 1 µg DNA). The incubation time, temperature and the buffer used in different reactions were according to the manufacturer`s

recommendations. The fragments obtained were analyzed by agarose gel electrophoresis or directly isolated using anion-exchange chromatography (Qiagen).

T4-DNA ligase was used to ligate DNA fragments; linearized DNA vector (50-200 ng) and 2-5 times molar concentration excess of DNA fragment(s) to be inserted, were incubated (in a 20 µl reaction) with 2 µl of 10x ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 5% (w/v) PEG-8000, 1 mM DTT, 1 mM ATP, pH 7.6), and 1 U T4-DNA ligase (Gibco-BRL). The reactions were performed at 14°C for 12 h or at RT for 3 h. The reactions were stopped by inactivating the enzyme (10 min, 65°C). The ligated DNA was used to transform *E. coli* competent cells.

2.1.5. Preparation and transformation of *E. coli* competent cells

Preparation of competent cells

For preparation of competent cells for electroporation, a single colony of the corresponding *E. coli* strain was inoculated into 5 ml of LB-medium and grown (ON, 37°C) under moderate shaking conditions. In the morning, 1 l LB-medium was inoculated with the overnight grown *E. coli* cells. The cells were grown further until they reached the logarithmic growth phase (OD_{578} = 0.5). After keeping them on ice for 30 min, the cells were harvested by centrifugation (4,400xg, 5 min, 4°C) and washed subsequently with 500 ml, 250 ml, and 50 ml 10% (v/v) glycerol. The competent cells were finally resuspended in 500 µl 10% (v/v) glycerol, aliquoted, and stored at – 80°C.

For the $CaCl_2$ transformation method, a culture of 1 l competent cells were grown to OD_{578} = 0.5 as above. The culture was the left on ice for 10 min and the cells were harvested by centrifugation (2,800xg, 10 min) under sterile conditions. The pellet was resuspended in 100 ml 50 mM $CaCl_2$ (sterile solution stored on ice) and left again on ice for 30 min. Then, the cells were resuspended in 8 ml 50 mM $CaCl_2$, sedimented and finally resuspended in 2 ml 75% glycerol (v/v), aliquoted, and stored at – 80 °C.

Transformation via electroporation

E. coli competent cells (40 µl) were incubated with 1-5 µl ligation mixture for 30 sec on ice. The suspension was transferred to an ice-cold cuvette and the cuvette was introduced in an electroporation apparatus, Gene Pulser (BioRad) (2.5 kV; 400 ohm; 25µF; time constant 8-9 ms). After a brief application of a high electric voltage to the cells, the suspension was diluted with 800µl LB-medium, and incubated for 30-60 min at 37°C under moderate shaking conditions. The transformed cells were harvested by centrifugation and plated on LB-medium plates supplemented with the appropriate antibiotic. The plates were incubated ON at 37°C.

Transformation via $CaCl_2$ method

For the $CaCl_2$ method, the competent cells were incubated on ice for 30 min with 1-5 µl ligation mixture. After a heat shock (2-5 min, at 42°C) the cells were incubated again on ice for 5 min. Then, the cells were harvested by centrifugation, resuspended in LB-medium, and incubated (40-50 min, 37°C) under moderate shaking conditions. The transformation was completed as described above for the electroporation method.

2.1.6. DNA purification and analysis

DNA fragments were separated by electrophoresis in a horizontal agarose gel (0.8-3%) according to their molecular weight. The samples were mixed with loading buffer (6% (v/v) glycerol, 0.05% bromphenolblue, 0.05% xylencyanol) and electrophoresis was performed in TAE-buffer (4.84 g/l Tris-Base, 1.14 ml/l acetic acid, 1 mM EDTA, pH 8.0). The agarose solution contained 0.5 µg/ml ethidium bromide, to allow visualization of DNA in gel under UV light.

DNA fragments required for further work were cut out from the gel with a clean scalpel and DNA was extracted from the gel and isolated using anion-exchange chromatography kit (Qiagen).

To determine the DNA concentration, the absorption of DNA solutions was measured at 260 nm. An OD of 1.0 corresponds to a concentration of 50 µg/ml of double stranded DNA, 33 µg/ml mono stranded DNA, 40 µg/ml RNA or 20 µg/ml oligonucleotides.

2.1.7. Cloning

Plasmids used:

Plasmid	Reference
pGEM4-AAC	Endres *et al.,* 1999
pGEM4-BCS1wt	Fölsch *et al.,* 1996
pGEM4-BCS1Δ65	Fölsch *et al.,* 1996
pGEM4-BCS1Δ82	Fölsch *et al.,* 1996
pGEM4-BCS1(1-86)-DHFR	Fölsch *et al.,* 1996
pGEM4-BCS1(1-126)-DHFR	Fölsch *et al.,* 1996
pGEM4-BCS1(1-126)-DHFRΔTM	This thesis
pGEM4-BCS1(84-126)-DHFR	This thesis
pGEM4-BCS1(66-86)-DHFR	This thesis

pGEM4-BCS1-(CoxIV)-BCS1	This thesis
pGEM4-BCS1-(CytC1)-BCS1	This thesis
pGEM4-BCS1-(Su9)-BCS1	This thesis
pGEM4-DHFR	Gaume *et al.,* 1998
pGEM4-Su9(1-69)-DHFR	Stan *et al.,* 2000
pGEM4-DHFR-BCS1(1-250)-DHFR	This thesis
pQE60-DHFR	Gaume *et al.,* 1998
pQE60-Su9(1-69)-barnase	This thesis
pQE60-Su9(1-69)-DHFR	Stan *et al.,* 2000

Cloning strategies

pGEM4-BCS1(1-126)-DHFR ΔTM

The DNA sequence encoding the first 126 amino acid residues of BCS1, but without the transmembrane domain, was amplified by PCR using the pGEM4-BCS1ΔTM as template (Fölsch *et al.,* 1996). The following primers were used:

Primer N-terminal (containing an *Eco*RI cutting site):

5`-CCC CGG ATC CTG ATT TGT CCT TCG ACT GAA-3`

Primer C-terminal (containing a *Kpn* I cutting site):

5`AAA AGG ATC CTC CAT TGT CAT GTT GTA TGT AGT T-3`.

The PCR product was digested with *Eco*RI and *Kpn* I and subcloned into the *Eco*RI /*Kpn* I cutting sites of a pGEM4 vector containing already the DHFR encoding sequence inserted between *Bam*HI/*Hind* III cutting sites. Three amino acids (RGS) were introduced by the cloning procedure into the joining region between the BCS1 gene part and the DHFR gene.

pGEM4-BCS1(84-126)-DHFR

The DNA sequence encoding amino acid residues 84-126 of BCS1 was amplified by PCR using the pGEM4-BCS1(1-126)-DHFR as a template. The following primers were used:

N-terminal primer (containing an *Eco*RI cutting site):

5`-AAA GAA TTC ATG ATT GTC GAC TTA GAG ATT CAG TCG AAG-3`

C-terminal primer (containing a *Sac* I cutting site):

5`-TTT AAG CTT GTG GTG GTG GTG GTG GTG GTG GAC-3`.

The PCR product was digested with *Eco*RI and *Sac* I and subcloned into the *Eco*RI /*Sac* I cutting sites of a pGEM4 vector containing already the DHFR encoding sequence inserted between *Sac* I/*Hind* III cutting sites.

pGEM4-BCS1(66-86)-DHFR

The DNA sequence encoding amino acid residues 66-86 of BCS1 was amplified by PCR using pGEM4-BCS1wt as a template. The following primers were used:

N-terminal primer (containing an *Eco*RI cutting site):

5`-CCC GAA TTC ATG GCT GTA GCT AGA TCC GGT ATA ATA AAA-3`

C-terminal primer (containing a *Kpn* I cutting site):

5`-AAA GGT AAC GTC GAC AAT CAT TTG TCG GTA TAG GAC-3`.

The rest of the cloning procedure was done as described for the BCS1(1-126)-DHFR ΔTM construct.

pGEM4-BCS1-(Su9)-BCS1

As the first stage, the pGEM4-BCS1(1-69)-Su9(1-48)-DHFR was constructed. The DNA sequence encoding amino acids residues 1-69 of BCS1 was amplified by PCR using pGEM4-BCS1wt as a template and the following primers, both containing an *Eco*RI cutting site:

N-terminal primer: 5`-AAA AGA ATT CAT GTC GGA TAA GCC AT TGA CAT A-3`

C-terminal primer: 5`-AAA AGA ATT CTA AAC CTG TAC CAA GGA TCA TAA G-3`.

The DNA product was digested on both sides with *Eco*RI and inserted into the *Eco*RI cutting site of a pGEM4 vector that already contained the pSu9(1-48)-DHFR coding sequence (the *Eco*RI cutting site was located upstream of this encoding sequence). Next, the sequence containing the DHFR gene was released from the vector by digestion with *Bam*HI/*Hind* III and was replaced with the PCR product encoding residues 84-458 of BCS1. This latter product was obtained using pGEM4-BCS1wt as a template and the following primers:

N-terminal primer (containing a *Bam*HI cutting site):

5`-AAA GGA TCC ATT GTC GAC TTA GAG ATT CAG TCG AAG-3`

C-terminal primer (containing a *Hind* III cutting site):

5`-AAA AAG CTT CTA GAA AAT ATG ATT AGC GTT CCG TAA GCT-3`.

pGEM4-BCS1-(CoxIV)-BCS1

First, a DNA sequence encoding amino acid residues 84-458 of BCS1, obtained as described above, was introduced into the *Bam*HI/*Hind* III cutting sites of an empty pGEM4 vector. Next, DNA sequence encoding amino acids residues 1-22 of CoxIV was amplified by PCR, using pGEM4-Cox IV-DHFR as template.

The following primers were used:

N-terminal primer (containing a *Kpn* I cutting site):

5`-AAA GGT ACC ATG CTT TCA CAT CGT CAA TCT ATA AGA TTT-3`

C-terminal primer (containing a *Bam*HI cutting site):

5`-AAA GGA TCC TCT AGA GCT ACA CAA AGT TCT TGT-3`.

This DNA sequence was digested with *Kpn* I/*Bam*HI and inserted into the *Kpn* I/*Bam*HI cutting sites of the pGEM4-BCS1(84-458). Finally, the DNA sequence encoding the amino acid residues 1-68 of BCS1 was introduced into the *Eco*RI/*Kpn* I sites of the above obtained vector. For *in vivo* studies, the pGEM4-BCS1-(CoxIV)-BCS1 was digested with *Eco*RI/*Hind* III and ligated into the yeast expression vector pYX142 cut with the same enzymes.

pGEM4-BCS1-($CytC_1$)-BCS1

The DNA sequence encoding amino acid residues 273-287 of Cytochrome c_1 was amplified by PCR using pGEM4-Tim23-$CytC_1$(248-309) as a template. The following primers were used:

N-terminal primer (containing a *Bam*HI cutting site):

5`-AAA AGG ATC CAC GGT GAT AAT CTT ATC-3`

C-terminal primer (containing a *Bgl*II cutting site):

5`-AAA AAG ATC CCC AGA TAG ATA GCA A-3`.

The PCR product was digested with the corresponding enzymes and inserted between the *Bam*HI/*Bgl*II cutting sites of pGEM4-BCS1ΔTM plasmid. For *in vivo* studies, the pGEM4-BCS1-($CytC_1$)-BCS1 was digested with *Bam*HI/*Bgl*II and ligated into the yeast expression vector pYX142 cut with the same enzymes.

pGEM4-DHFR-BCS1(1-250)-DHFR

The DNA sequence encoding amino-acid residues 1-250 of BCS1 placed at the C-terminal of DHFR, was amplified by PCR using pGEM4-DHFR-BCS1 as template and the following primers:

N-terminal primer (containing an *Eco*RI cutting site):

5`-AAA GAA TTC ATG GTT CGA CCA TTG AAC TGC ATC GTC-3`

C-terminal primer (containing a *Kpn* I cutting site):

5`-AAA GGT ACC TTT GCC GTT CTT CAT AAA ATC ATA AAC-3`.

The obtained DNA sequence was introduced into the *Eco*RI/*Kpn* I cutting sites of a pGEM4 vector that already contained DHFR encoding sequence between the *Bam*HI and *Hind* III cutting sites.

pQE70-Su9(1-69)-barnase

The DNA fragment encoding this construct was amplified using pQE60-Su9(1-69)-barnase as template and the following primers:

N-terminal primer (containing a *Bam*HI cutting site):

5`-TTG GAT CCA TGG CCT CCA CTC GTG TCC TCG CC-3`

C-terminal primer (containing a *Hind* III cutting site and introducing a 6xHis tag at the C-terminal of the protein):

5`-TTT TTA AGC TTA GTG GTG GTG GTG GTG GTG GTG GCA TCT GAT TTT TGT AAA GGT CTG ATA-3`.

After digestion with the corresponding enzymes, the construct was ligated into the pQE70 expression vector and transformed into the *E. coli* BL21(DE3) strain.

2.2. *Genetic Methods*

2.2.1. *E. coli:* Culture and Media

*E. coli strains used***:** MH1, XL1-Blue, BL21(DE3).

Media for E. coli

LB-medium: 1% Bacto-Tryptone, 0.5% Yeast extract, 1% NaCl;

LB^{Amp}-medium: LB-medium supplemented with 100 µg/ml ampicillin.

The described media were used for preparing liquid cultures. To prepare plates with solid media, 2% w/v bacto-agar was added. Bacto-agar, glucose and media were autoclaved separately. The appropriate antibiotics were added after the media were chilled to 50°C.

2.2.2. *N. crassa:* Culture and Media

N. crassa strains used: wt 74A, GR 107 (Tom22 contains a 6xHis tag at the C-terminal).

Media for N. crassa

Trace elements solution: 50 g Citric acid, 50 g $ZnSO_4$, 10 g $Fe((NH_4)\ SO_4)$, 2.5 g $Cu\ SO_4$, 0.5 g $MnSO_4$ x H_2O, 0.5 g H_3BO_3 (water free), 0.5 g Na_2MoO_4 in 1 l H_2O.

Biotin solution: 100 ml ethanol, 100 ml H_2O, 20 mg biotin.

50x Vogel's minimal medium: 150 g Na_3-Citrate x H_2O, 250 g KH_2PO_4, 100 g NH_4NO_3, 10g $MgSO_4$, 5g $CaCl_2$, 5 ml trace elements-solution, 2.5 ml biotin solution in 1 l H_2O.

Complete medium: 2% 50x Vogel's minimal medium, 1% (w/v) glycerol, 1% (w/v) sugar, 0.2% (w/v) yeast extract; 0.1% (w/v) caseinhydrolysat.

N. crassa growth

N. crassa growth was performed as previously described (Davis and Serres, 1970).

Obtaining the conidia

Conidia growth was done in 250 ml Erlenmeyer flasks on complete medium supplemented with 2% agar, so that the mycels could grow upwards. The medium was inoculated with silicate dried hyphae and incubated for 3-7 days in the dark, followed by further 3-5 days at RT, and daylight, to stimulate conidia formation. To collect the conidia, 50 ml sterile H_2O was added and the suspension was filtrated through sterile cotton. The concentration of conidia suspension was determined by counting under the light microscope.

Growth of hyphae

To isolate small scale mitochondria from *N. crassa* wild type 74A strain, 1 l of MIN-medium (930 ml H_2O, 20 ml 50x Vogel's minimal medium (Vogel, 1964) and 40 ml 1.4 M sucrose; all the components autoclaved separately) was inoculated with 10 ml conidia suspension (10^8 cells/ml) and incubated (15 h, 25°C) under light and aeration. The hyphae were collected by filtration. To obtain large amounts of hyphae, 100 l cultures (containing 2 kg sucrose and 2 l 50x Vogel's minimal medium) inoculated with 1 l conidia (10^8 cells/ml) were incubated (24 h, 25°C) under light and aeration condition. For growing the *N. crassa* GR107 strain, the medium was supplemented with 1.3 mM histidine. The hyphae were collected and after wet weight determination, used for mitochondria or TOM complex isolation.

2.2.3. *S. cerevisiae*: Culture and Media

S. cerevisiae strains used:

Strain	Genotype	Reference
W303-1A	MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100 Isogenic with RS 190 (ATCC 208354)	R. Rothstein, Department of Human Genetics, Columbia University, New York
W303-1B	MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100	R. Rothstein, Department of Human Genetics, Columbia University, New York
W334-a	MATa leu2 ura3-52	Hovlan *et al.,* 1989
D 273-10B	ATCC246557 MATα Mal (rho+)	German Collection for Microorganisms
BY 4743	Mata/α, his3/his3, leu2/leu2, ura3/ura3, met15/MET15, lys2/LYS2	Brachmann *et al.,* 1998
Δbcs1	W303-1A, bcs1::HIS3	Nobrega *et al.,* 1992

Δbcs1	Mata/α, his3/his3, leu2/leu2, ura3/ura3, met15/MET15, lys2/LYS2, bsc1:: kanMX4/ (homozygous diploid deletion strain of bcs1 in BY4743)	Giever *et al.,* 2002

Media for S.cerevisiae

YP-medium: 1% Yeast extract, 2% peptone, pH 5.0 (adjusted with HCl);

YPD-medium: YP-medium supplemented with 2% glucose;

YPG-medium: YP-medium supplemented with 3% glycerol;

SC-medium: 0.17% (w/v) Yeast Nitrogen Base, 0.5% (w/v) ammonium sulphate, 1.5 g/l "Drop-out mix" powder (mix containing equal weight of all amino acids; for selecting one auxothophic marker, the corresponding amino acid was left out), 2% glucose or 3% glycerol as carbon source.

The described media were used for preparing liquid cultures. To prepare plates with solid media, 2% w/v bacto-agar was added. Bacto-agar, glucose, and media were autoclaved separately.

S. cerevisiae growth

S. cerevisiae growth was performed as previously described (Sambrook *et al*., 1989) in YPD complete medium or, when a selection on the auxotrophic marker was necessary, on SD-medium. The cells were incubated at 30°C, under shaking conditions.

To determine the growth characteristics of yeast strains, a dilutions assay was performed. The strains were grown in liquid culture to the logarithmic phase. Equal amounts of cells (0.5 OD_{578} units) from every culture were isolated, resuspended in 500 µl H_2O, and a series of 1:10 dilutions were made; 5 µl from each dilution was spotted on agarose plates. The results could be seen after 2-4 days incubation at the appropriate temperature.

Transformation of S. cerevisiae (lithium acetate method)

The corresponding yeast strain was grown ON in YPD-medium and diluted the next morning in 50 ml medium, to an OD_{578} of 0.2. The cells were further grown till they reached an OD_{578} of 0.8, transferred to a sterile centrifuge tube, and harvested by centrifugation (1,000xg, 3 min, RT). After washing with 25 ml of sterile H_2O, the cells were harvested under the same conditions, resuspended in 1 ml 100 mM lithium acetate and transferred to an Eppendorf tube. The sample was centrifuged again (7,500xg, 15 sec) and the cells were resuspended in 400 µl 100 mM lithium acetate. For each transformation 50 µl suspension of cells was centrifuged (7,500xg, 5 min) and the supernatant was

removed. The following mixture was added to the cells: 240 µl PEG 3350 (50% v/v), 36 µl 1 M lithium acetate, 5 µl single stranded Salmon sperm DNA (10 mg/ml; previously incubated for 5 min at 95°C), 50 µl H_2O containing 0.1-10 µg DNA to be transformed. The mix was vortexed for 1 min and incubated for 30 min at 30°C, with moderate shaking, followed by 20-25 min incubation at 42°C.

The cells were harvested by centrifugation (3,000-4,000xg), resusepended in 800 µl YPD-medium, and further incubated for 2 h at 30ºC. After harvesting, the cells were resuspended in a small volume of medium, and spread on selective solid media. The plates were incubated for 2-4 days at 30°C to recover transformants.

2.3. *Cell Biological Methods*

2.3.1. Isolation of mitochondria from *S. cerevisiae*

Mitochondria were isolated from *S. cerevisiae* following a previously described method (Herrmann et al., 1994). Yeast cells were cultivated to OD_{578} of 1-2, harvested by centrifugation (4,400xg, 5 min, RT), washed with H_2O and resuspended to a final concentration of 0.5 g/ml in DTT buffer (100 mM Tris/SO_4, 10 mM dithiotreitol (DTT), pH 9.4). The cell suspension was incubated for 15 min at 30°C with moderate shaking, followed by a new centrifugation step and resuspended in 100 ml of 1.2 M sorbitol. To digest the cell wall, the cells were resuspended in Zymolyase buffer (1.2 M sorbitol, 20 mM potassium phosphate/KOH, pH 7.4) to a concentration of 0.15 g/ml and incubated with 3 mg Zymolyase /g wet weight for 30-45 min at 30°C, under moderate shaking conditions.

To test the cell wall digestion (obtaining of spheroplasts), 50 µl cell suspension was diluted with 2 ml H_2O or into a solution of 1.2 M sorbitol. Formation of spheroplasts was complete when the OD of the H_2O dilution was 10-20% of the OD of the sorbitol dilution. The solution of spheroplasts in pure H_2O becomes clear because spheroplasts burst under these conditions. All the subsequent steps were performed at 4°C.

The spheroplasts were isolated by centrifugation (3,000xg, 5 min, 4°C), resuspended (0.15 g/ml) in homogenizing buffer (0.6 M sorbitol, 10 mM Tris/HCl, 1 mM EDTA, 0.2% (w/v) BSA, 1 mM PMSF, pH 7.4), and homogenized 10 times in a Dounce-Homogenisor. The cell remnants and unopened cells were sedimented by double centrifugation (2,000xg, 5 min, 4°C). The supernatant was centrifuged (17,400xg, 12 min, 4°C) and the sedimented mitochondria were resuspended in SEM buffer (10 mM MOPS/KOH, 250 mM sucrose, 1 mM EDTA, pH 7.4) and separated again from cell

remnants (2,000xg, 5 min, 4º). The mitochondria were sedimented again as above. Finally mitochondria were resuspended in a small volume of SEM buffer, to a concentration of 10 mg/ml protein, aliquoted, frozen in liquid nitrogen, and stored at – 80ºC till use.

2.3.2. Crude isolation of mitochondrial membranes from *S. cerevisiae*

Cells corresponding to 10 OD units were harvested by centrifugation (3,000xg, 5 min) and washed with H_2O. The cells were resuspended in SEM buffer and 0.3 g glass beads (diameter 0.3 mm) were added. The samples were vortexed 4 times for 30 sec each, with 30 sec breaks in between (during this break the samples were incubated on ice). After centrifugation (1,000xg, 3 min) the supernatant was transferred to a new tube and centrifuged again (10,000xg, 10 min, 4°C). The pellets containing mitochondria were resuspended in 25 µl sample buffer (60 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.02% (w/v) bromphenolblue, 5% β-mercaptoethanol), shaken for 10 min at RT, and analyzed by SDS-PAGE.

2.3.3. Isolation of mitochondria from *N. crassa*

Hyphae (10 g wet weight), grown as describe above, were mixed with 15 g quartz sand and 20 ml SEMP (buffer SEM supplemented with 1 mM PMSF), and ground in a mortar for 1-2 min. The mix was centrifuged twice (3,000xg, 5 min, 4ºC) to get rid of cellular residues, and the mitochondria were sedimented (17,000xg, 12 min), resuspended in 30 ml SEM, sedimented again and finally resuspended in 0.5-1 ml SEM. The mitochondria were used for import experiments within 1 h as storage in the freezer ruptures the outer membrane.

For a large scale preparation of mitochondria, necessary for preparation of outer membrane vesicles, the same protocol was followed, with small modifications. Hyphae from a 100 l culture (1-2 Kg) were mixed with 3 l SEMP and 1,5-3 Kg quartz sand, homogenized for 3x10 sec in a Warnig blender, and passed through a grill mill (Sebald *et al.,* 1979). The mitochondria were isolated from the homogenate by differential centrifugation as above. The two last centrifugation steps were for 50 min at 17,700xg. Before and after the last centrifugation step, the mitochondria were resuspended in SM buffer (250 mM sucrose, 10 mM MOPS/KOH, pH 7.2).

2.3.4. Isolation of outer membrane vesicles (OMV) from *N. crassa*

OMV were isolated as previously described (Mayer *et al.,* 1993). Mitochondria isolated from *N. crassa* were resuspended in hypotonic swelling buffer (5 mM potassium

phosphate, 1 mM EDTA, 1 mM PMSF, pH 7.2) to a concentration of 2 mg/ml and incubated for 30 min at 4°C. The mitochondrial membranes were reisolated by centrifugation (17,700xg, 4°C), resuspended again and incubated in swelling buffer, (5 min, 37°C), and finally transferred into an automatic Glass-Teflon-Homogenisator (60 ml per machine) and homogenized (40 min, 0°C), in order to separate the outer membranes from mitoplasts (mitochondria with disrupted outer membrane). For the separation via sucrose gradient centrifugation, 20 ml homogenate were layered under 10 ml of 0.9 M sucrose and 9 ml 0.25 M sucrose in EMP (1 mM EDTA, 10 mM MOPS, 1 mM PMSF, pH 7,0) and separated during 1 h centrifugation (141,000xg, 4ºC), in a Beckmann SW28 rotor. The intact mitochondria, mitoplasts, and inner membrane fragments were found at the bottom, and the OMV collected in the "intermediate density fraction", between the 0.25 M and 0.9 M sucrose gradient layers. This material was collected and adjusted to a 0.9 M sucrose concentration with 2 M sucrose. In a second centrifugation step gradient, 15 ml samples were layered under 21 ml 0.72 M sucrose in EMP and 3 ml EMP (no sucrose), and centrifuged (10 h at 141,000xg, 4°C). The OMV were collected between 0.72 M and the upper gradient layers. OMV were diluted 1:2 with 10 mM KOAc buffer, pH 7.0, sedimented via centrifugation (141,000xg, 30 min, at 4°C), resuspended in a small volume of the same buffer, frozen in liquid nitrogen, and stored at –80°C till use.

2.3.5. Isolation of TOM complex from *N. crassa*

Isolation of the TOM holo complex was done as describe before (Künkele *et al.,* 1998). The OMV for *N. crassa* strain GR 107 were resuspended to a final concentration of 1 mg/ml in solubilization buffer (50 mM KOAc, 10 mM MOPS, 20% glycerol, 1.25% digitonin, 1 mM PMSF, pH 7.0) and incubated (30 min, 4°C) under moderate shaking conditions. The undissolved material was sedimented (226,200xg, 30 min) and the supernatant was loaded onto a Ni-NTA column at a flow rate 1 ml/min (1 ml Ni-NTA beads/10 mg OMV). The column was washed with 2 column-volumes of solubilization buffer containing 1.25% digitonin and with 20 column-volumes of solubilization buffer containing 0.5% digitonin. Elution of the unspecifically bound material was done with a gradient concentration from 0 to 60 mM imidazole and the elution of the bound TOM complex was performed with solubilization buffer containing 0.5% digitonin and 300 mM imidazole.

Isolation of TOM core complex from *N. crassa* mitochondria was performed as described before (Ahting *et al.,* 1999). Mitochondria (5 g) were resuspended in solubilization buffer (50 mM KOAc, 10 mM MOPS, 20% glycerol, 1% Dodecyl maltoside

(DDM), 1 mM PMSF, pH 7.0) to a final concentration of 10 mg/ml, and incubated (30 min, 4°C) under moderate shaking conditions. The undissolved material was sedimented (226,200xg, 30 min) and the supernatant was loaded at a flow rate of 3 ml/min onto a Ni-NTA column previously equilibrated with 1.5 column-vol of solubilization buffer. The column was washed with 8 column-vol of DDM-washing buffer (50 mM KOAc, 10 mM MOPS, 20% glycerol, 0.1% DDM, 30 mM imidazole, 1 mM PMSF, pH 7.0) at the same flow rate. The bound material was eluted with 50 ml DDM-elution buffer (50 mM KOAc, 10 mM MOPS, 20% glycerol, 0.1% DDM, 300 mM imidazole, 1 mM PMSF, pH 7.0). For a second purification step the material eluted from the Ni-NTA column was loaded with a flow rate of 1 ml/min onto an anion-exchange column (Pharmacia Biotech), previously equilibrated with 2 column-vol of Resource buffer A (50 mM KOAc, 10 mM MOPS, 20% glycerol, 1% DDM, 1 mM PMSF, pH 7.0), 2 column-vol of Resource buffer B (50 mM KOAc, 10 mM MOPS, 20% glycerol, 1% DDM, 1 M KCl, 1 mM PMSF, pH 7.0), and 5 column-vol of Resource buffer A. The bound material was eluted with 10 ml linear gradient of 0-50% Resource buffer B, at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. The TOM core complex eluted at 250 mM KCl.

2.3.6. Isolation of lipids from outer membrane vesicles of *N. crassa*

Isolation of lipids from outer membrane vesicles of *N. crassa* followed a previously described method (Bligh and Dyer, 1959). For the extraction of up to 1000 nmol of lipid phosphorus, 1 vol of OMV (containing 50-100 mg protein), was mixed with 1 vol of 0.1 M HCl, 2 vol of methanol (or more, till the solution was clear), and 1 vol chloroform; after 10 sec of vortexing, 1 vol 0.1 M HCl and 1 vol chloroform were added and the probes were vortexed again till two phases were obtained. The mix was centrifuged (1,100xg, 5 min) and the subphase (chloroform phase) was collected with a Pasteur pipette, washed twice with 1 vol H_2O, and reisolated by centrifugation (the probes were supplied with gaseous nitrogen before each centrifugation step, in order to avoid lipid oxidation). The chloroform phases collected from the centrifugations were mixed with equivalent volume of deionized H_2O and centrifuged again. Next, the chloroform was evaporated in a rotating “evaporator” till a lipid film was obtained. The lipid film was dissolved either in chloroform/methanol for lipid analyses, or in the appropriate buffer for performing experiments with the TOM complex.

2.3.7. Quantification of phosphorus

Samples containing lipids or detergents were dried, in glass tubes, at 180°C, supplied with 300 µl 70% perchloric acid and incubated again (1.5 h, 180°C) in a heating block. After chilling the tubes to RT, 3 ml ammonium molybdate solution, 120 µl "Fiske and Subbarow" reagent were added and the samples were vortexed and boiled for further 15 min at 100°C. The intensity of the blue color of the probes was proportional to the quantity of the contained phosphorus; the OD was measured at 830 nm. The values were compared with a standard curve obtained with KH_2PO_4 solutions.

For preparing the ammonium molybdate solution, 11 g of ammonium heptamolybdate x 4 H_2O were dissolved in 100 ml 98% H_2SO_4, and the solution was filled with distilled H_2O up to 5 l.

For preparing the "Fiske and Subbarow" reagent, 27.36 g $Na_2S_2O_5$, 1 g Na_2SO_3, 0.5 g 1-amino-2-naftol-4-sulfonic acid, were dissolved ON in 200 ml distilled H_2O. After filtration, the reagent was stored in the dark at RT.

2.3.8. Purification of immunoglobulin G (IgG)

Antiserum (4 ml) was centrifuged, (20,000xg, 20 min, 4°C) and the white surface film of aggregated lipids was removed. The antiserum was diluted with 10 ml of buffer A (100 mM KPi pH 8.5), filtrated, and was loaded onto a 5 ml Protein A-Superose column (Pharmacia), previously equilibrated with buffer A. After washing the column with 5 column volumes of buffer A, the bound IgGs were eluted with buffer B (100 mM Citrate/NaOH, pH 3.0). The eluate was immediately neutralized with 2 ml 2 M Tris/HCl, pH 8.0, dialyzed ON against 5 l of H_2O and concentrated via lyophilization. The IgGs were resuspended in 10 mM MOPS/KOH, pH 7.2 to a final protein concentration of 10-50 mg/ml, aliquoted, and stored at – 20ºC till use.

2.3.9. Purification of recombinant proteins over-expressed in *E. coli*

Purification of recombinant MPP out of *E. coli* was done as described before (Luciano *et al.,* 1997). The *E. coli* strain containing the MPP gene in pVG18 vector was grown (ON, 37°C) in a small volume of LB^{Amp}-medium. The next morning the culture was diluted to an OD_{578} of 0.1, further incubated till it reached an OD_{578} of 0.6, and induced for 2 h with 1 mM isopropil-β,D-tiogalactopyranoside (IPTG). Bacteria were harvested by centrifugation (3,000xg, 5 min), washed with H_2O, resuspended in 20 ml of buffer (50 mM NaCl, 50 mM Na-phosphate, 0.025% Lysozyme, 1 mM PMSF, pH 8.0) and incubated for 20 min at 0°C in order to degrade the cell walls and to obtain spheroplasts. The

spheroplasts were reisolated via centrifugation (3,000xg, 5 min), washed with the same buffer, but without Lysozyme, and resuspended in 20 ml of a buffer containing 50 mM NaCl, 50 mM Na-phosphate, 10% glycerol, 10 mM imidazole, pH 8.0 and protease inhibitors. The suspension was left on ice and sonicated 5 times for 30 sec with a Branson sonicator 450 (30% pulse; out put 3; 80% duty cycle). The sonicated suspension was centrifuged (39,000xg, 15 min) and the supernatant was applied onto a Ni-NTA column with a flow rate of 0.3 ml/min. The Ni-NTA beads were previously washed with 20 ml of buffer A. The bound proteins were eluted with 30 ml buffer B (50 mM NaCl, 50 mM Na-phosphate, 10% glycerol, 500 mM imidazole, pH 8.0).

Chemical amounts of Su9(1-69)-DHFR, Su9(1-45)-DHFR and Su9(1-69)-barnase were purified in the same manner, with minor modifications. Buffer A and B contained 300 mM NaCl and the corresponding *E. coli* strains were grown on LB^{Amp}-media containing 50µg/ml Kanamycin.

For purification of the recombinant cytosolic domain of Tom70 (*S. cerevisiae*), the transformed *E. coli* strain containing the over-expression vector pRSETA-Tom70 (Young *et al.,* 2003) were grown at 37ºC, under shaking conditions, on LB^{Amp}-medium, till they reached an OD_{578} of 0.5. After supplementing the medium with 0.5% glucose and 0.8 mM IPTG, the culture was shifted to 18°C for 16 h. Collection of the cells was done like for MPP purification. The purification succeeded in two steps. The first step was done via Ni-NTA chromatography. In order to release the DnaK contaminations, after loading the sample, the Ni-NTA column was washed with Buffer A supplemented with 5 mM MgOAc. The bound material was eluted with buffer B (20 mM K-Phosphate and 300 mM imidazole). Fractions with proteins were pooled and a second purification step via anion-exchange chromatography was done. For equilibration of the 1 ml HiTrap Q column, 20 mM K-Phosphate buffer/KOH, pH 7.5 was used. The elution was done with a gradient between 0 and 1 M NaCl. The cytosolic domain of Tom70 was released at a concentration of 250 mM NaCl.

2.4. *In vitro import experiments*

2.4.1. Synthesis of radioactive labelled proteins *in vitro*

Transcription /Translation in a cell-free system

For *in vitro* synthesis of ^{35}S labelled proteins, the constructs contained in pGEM4 plasmid were first transcribed in mRNA using SP6-RNA-polymerase (Melton, 1984; Sambrook, 1989). Transcription mixture (100µl) contained: 10-20 µg DNA, 20 µl 5x transcription buffer (200 mM Tris/HCl, 50 mM $MgCl_2$, 10 mM spermidine, pH 7.5), 10 µl

0.1 M DTT, 4 µl RNasin nuclease inhibitor (40 U/µl), 20 µl 2.5 mM rNTP, and 5.2 µl 2.5 mM M7G(5`)ppp(5`)G. After adding 3 µl of SP6-Polymerase (25 U/ml) the mixture was incubated for 1 h at 37°C. The RNA was precipitated by adding 10 µl of LiCl (10 M) and 300 µl ethanol (96%), centrifuged, and washed with 70% ethanol. After ethanol evaporation at RT, the RNA was resuspended in H_2O supplemented with 1 µl RNasin (40 U/µl), aliquoted and kept at – 80°C till use.

For translation using rabbit reticulocyte lysate, the following mix was incubated for 60 min at 30°C: 25 µl RNA, 3.5 µl amino acid mix (without methionine), 7 µl 15 mM MgOAc, 12 µl ^{35}S (10 mCi/ml) and 100 µl rabbit reticulocite lysate (Promega). At the end of the translation reaction 5 mM of cold methionine and 250 mM sucrose were added.

TNT Coupled reticulocyte lysate system

As alternative to the two separate steps of transcription and translation, the TNT Coupled reticulocyte system offers a possibility for simultaneously performing the two tasks. TNT mix (50µl) contains: 25µl TNT rabbit reticulocyte lysate (Promega), 2 µl TNT reaction buffer, 1 µl TNT RNA polymerase (SP6, T3 or T7), 1 µl amino acid mix, 2 µl ^{35}S methionine (10 mCi/ml), 1 µl RNasin ribonuclease inhibitor (40 U/µl), 2 µl DNA template (0.5 µg/µl). The TNT reaction was incubated for 90 min at 30°.

2.4.2. Import of preproteins into isolated mitochondria and binding of preproteins to outer membrane vesicles

Binding of precursor proteins to OMV was performed in buffer A (0.25 mg/ml bovine serum albumine (BSA), 20 mM KCl, 2.5 mM $MgCl_2$, 10 mM MOPS-KOH, pH 7.2) in the presence of 1 mM NADPH and 1 µM MTX, where indicated. At the end of the binding reactions, the OMV were washed with EM buffer (1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) containing the indicated concentrations of KCl. The OMV were sedimented (109,00xg, 20 min), solubilized in sample buffer and analyzed by SDS-PAGE.

Import of radiolabelled precursor proteins into *N. crassa* and *S. cerevisiae* mitochondria was performed in F5 import buffer (0.5% (w/v) BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM NADH, 10 mM MOPS-KOH, pH 7.2) or SI buffer (3% BSA (w/v), 0.5 M sorbitol, 50 mM HEPES-KOH, 80 mM KCl, 10 mM MgOAc, 2 mM KH_2PO_4, 2.5 mM EDTA, 2.5 mM $MnCl_2$, 2 mM ATP, 2 mM NADH, pH 7.2), respectively. The import reactions were performed at the indicated temperature and for various time periods. In some cases the mitochondria and OMV were protease treated before or after the import reaction. Protease treatment was performed by incubation with Proteinase K (PK) or trypsin for 15 min on ice, followed by addition of either 1 mM PMSF

or 20-fold trypsin inhibitor, respectively. At the end of import reactions mitochondria were sedimented (35,000xg, 10 min) and solubilized in sample buffer.

Imported and bound proteins were analyzed by SDS-PAGE, autoradiography and phosphorimaging (Fuji BAS 1500).

2.4.3. Generation of mitoplasts

To obtain mitoplasts, mitochondria resuspended in SI buffer were diluted 10 times with buffer containing 20 mM HEPES-KOH, pH 7.2. To check the disruption of the outer membrane, immunodecoration with antibodies against Cytochrome b_2 (soluble protein in the intermembrane space) was performed.

2.4.4. Carbonate extraction

Carbonate extraction was used to check whether a protein is inserted into a membrane or it is in a soluble form. For this purpose, mitochondria or mitoplasts were pelleted after import, via centrifugation, resuspended in buffer containing 10 mM HEPES-KOH, 100 mM Na_2CO_3, pH 11.5 and incubated for 30 min at 0ºC. The probes were then centrifuged for 35 min at 109,000xg. The soluble proteins were found in the supernatant and were precipitated with Trichloroacetic acid (TCA) and the proteins inserted/associated with the membranes were found in the pellet. The samples were analyzed by SDS-PAGE.

2.4.5. Co-immunoprecipitation

Samples (after import of radiolabelled precursors into mitochondria or binding to OMV) were dissolved in lysis buffer (3% BSA, 1% DDM, 250 mM sucrose, 1 mM EDTA, 150 mM KCl, 10 mM MOPS-KOH, pH 7.2) for 30 min, under mild shaking conditions. After a clarifying spin (15,000xg, 15 min), the supernatants were incubated for 1-3 h with antibodies that were pre-coupled to Protein A-Sepharose beads. After this incubation, the beads were washed with 500 µl lysis buffer, and finally with 500 µl $SEMK^{80}$ (SEM buffer supplemented with 80 mM KCl), 500 µl 10 mM Tris-HCl, pH 7.2. Bound proteins were eluted with sample buffer and analyzed by SDS-PAGE.

2.4.6. Screening of peptide libraries with soluble domains of Tom receptors

Screening of peptide libraries with soluble domains of Tom receptors was done as describe before (Brix *et al.*, 1999). Cellulose-bound peptide libraries were prepared by automated spot synthesis (Egan *et al.,* 1999; Kanaji *et al.,* 2000). Peptides of 13 amino acid residues and with an overlap of 10 residues, covering amino acid residues 1-126 of BCS1,

were linked to the cellulose membrane via (β-Ala)2 spacer. The peptide library was kindly provided by Prof. J. Schneider-Mergener (Berlin). The dry membranes were incubated once with methanol and three times with washing buffer (100 mM KCl and 30 mM Tris-HCl, pH 7.6) at RT for 10 min. For analysis of protein binding activity, the membranes were incubated with 150 nM of the soluble cytosolic domains of Tom20, Tom22, or Tom70 (kindly provided by Dr. J. Brix, Freiburg) in binding buffer (100 mM KCl, 5% (w/v) sucrose, 0.05% (v/v) Tween 20, 0.05% (w/v) BSA, and 30 mM Tris-HCl, pH 7.6) for 60 min at 25°C with gentle shaking.

After washing (3 min, RT) the peptide-bound proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using cathode buffer (75 mM Tris base, 120 mM 6-aminohexanoic acid, and 0.01% SDS) and anode buffers AI and AII (AI containing 90 mM Tris base and AII containing 300 mM Tris base). Tom proteins were detected with antibodies against the corresponding Tom component. Binding data were analyzed by scanning laser densitometry and quantified using TINA program.

2.4.7. Pull-down assay

To demonstrate a direct interaction between the BCS1 protein and Tom70 a pull-down assay was used. Purified recombinant cytosolic domain of Tom70 (2 μM) were incubated with Ni-NTA beads in buffer A (20 mM HEPES-KOH, 100 mM KOAc, 5% glycerol, 5 mM MgOAc, 2 mg/ml BSA, pH 7.5). After 30 min incubation at RT with moderate shaking, the unbound material was removed by washing the Ni-NTA beads with excess of buffer A. Radiolabelled proteins were then added and incubated with the beads in buffer A supplemented with 2 mM ATP, 5 μM MTX, and 1 mM NADPH for 30 min at 4ºC. The ATP was added to release the *in vitro* synthesized proteins from the chaperones present in the translation mixture. To avoid unspecific binding to Ni-NTA beads, MTX and NADPH were added to stabilize folded conformation of the DHFR domain. The unbound material was removed by washing with buffer A and the bound material was eluted from the beads by adding sample buffer supplemented with 300 mM imidazole. The bound material was analyzed by SDS-PAGE and autoradiography.

2.5. *Biochemical Methods*

2.5.1. Trichloroacetic acid (TCA) precipitation of proteins

Proteins from aqueous solutions were precipitated by adding 72% (w/v) TCA to a final concentration of 12.5%. The samples were incubated for 20 min on ice, and centrifuged (20 min, 30,000xg, 2°C). The precipitated proteins were washed with acetone

(kept at – 20ºC), and centrifuged again (30,000xg, 20 min). The protein pellet was dried for 10 min at RT and dissolved in sample buffer.

2.5.2. Ammonium sulphate precipitation of proteins

Aqueous solutions containing proteins were mixed with 2 vol of saturated solution of ammonium sulphate (4°C), to a concentration of 66%. The samples were incubated for 30 min at 4°C and centrifuged for 10 min, 30,000xg, at 4°C. The pellets containing the precipitated proteins were dissolved in the appropriate buffer.

To prepare the saturated ammonium sulphate solution, 76,7 g of ammonium sulphate was dissolved in 100 mM Tris/HCl pH 7.0 and chilled to 4°C, so that the ammonium sulphate crystals sediment.

2.5.3. Protein concentration determination

Protein concentrations were determined using Bradford assay (Bradford, 1976) and immunoglobulin G as standard (Bio-Rad). Protein solutions were diluted with 1 ml Bio-Rad reagent (dilution 1:5) and incubated for 10 min at RT. The absorbance was measured at 595 nm using a 1-cm-path length microcuvette. Protein concentration was calculated according to a standard curve.

2.5.4. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins were separated via one-dimensional vertical slab SDS-Polyacrylaminde gel electrophoresis (SDS-PAGE), under denaturing conditions, as described (Laemmli, 1970). The concentration of acrylamide and bis-acrylamide in the separating gel was chosen according to the molecular size of the proteins to be separated. Glass plates of 160 x140 mm and spacers of 1 mm thickness were used. The samples were dissolved in 25-40 µl sample buffer and incubated at 95°C for 3 min, before loading. The electrophoresis was performed at 25 mA for 3 h. Protein molecular weight standards were used.

Buffers for SDS-PAGE:

Stacking gel: 5% (w/v) acrylamide, 0.03% (w/v) bis-acrylamide, 60 mM Tris/HCl pH 6.8, 0.05% (w/v) APS, 0.25% (v/v) TEMED.

Separating gel: 10-16% (w/v) acrylamide, 0.07-0.3% (w/v) bis-acrylamide, 380 mM Tris/HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED.

Bottom gel: 20% acrylamide, 0.13% bis-acrylamide, 475 mM Tris/HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.25% (v/v) TEMED.

Sample buffer: 60 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.02% (w/v) bromphenolblue, 5% β-mercaptoethanol.

2.5.5. Blue-Native gel electrophoresis (BNGE)

For separation of proteins under non-denaturing (native) conditions, Blue-Native gel electrophoresis (BNGE) was used (Schagger, 1991). For this purpose, the mitochondria or OMV were pelleted, resuspended in 50 µl of Buffer N (containing the appropriate amount of detergent) and solubilized for 30-40 min at 4ºC. After a clarifying spin (30,000xg, 10 min), 5 µl of sample buffer were added to the soluble material, mixed for 3 min at RT and finally loaded onto a 6%-13% gel.

Buffers for BNGE:

Acrylamide 48%/Bis-acrylamide 1.5%: acrylamide 48 g, bis-acrylamide 1.5 g in 100 ml H_2O.

Acrylamide 30%/Bis-acrylamide 0.2%: acrylamide 300 g, bis-acrylamide 2 g in 1 l H_2O.

Gel-buffer: 1,5 M 6-amino-n-Capronic acid, 0,15 M bis Tris, pH 7.0.

10x sample buffer: 5% Coomassie-Brilliant-blue G250, 100 mM bis-Tris pH 7.0, 500 mM 6-amino-n-Capronic acid

Cathode-Buffer: 15 mM bis-Tris, pH 7.0, 50 mM Tricine, with or without 0.02% Coomassie-Brilliant-blue G250.

Anode-Buffer: 50 mM bis Tris, pH 7.0.

Buffer N: 20 mM Tris, 0.1 M EDTA, 50 mM NaCl, 1 mM PMSF, 10% glycerol, 0.1-1% digitonin or other detergent, pH 7.4.

The gel for BNGE was prepared and used on the same day. The samples were neither incubated at 96ºC nor frozen. The electrophoresis was performed at 4ºC. For the first step of electrophoresis, cathode buffer containing Coomassie-Brilliant-blue and anode buffer were used and the voltage was set for 1 h at 100V and than shifted to 500V. When the blue front had migrated to two thirds of the separation distance, the cathode buffer with Coomassie-Brilliant-blue was replaced by a cathode buffer without Coomassie-Brilliant-blue. Marker proteins like Apoferritin (440 kDa), Alcohol dehydrogenase (ADH), (monomer: 50 kDa, dimer: 100 kDa, trimer: 150 kDa), and bovine serum albumin (BSA), (monomer: 66 kDa, dimer: 136 kDa) were used.

2.5.6. Coomassie blue staining of SDS-Gels

After SDS-PAGE, the bottom and the staking gel were removed, and the separating gel was stained for 30 min, at RT, with solution containing 30% (v/v) methanol, 10% (v/v)

acetic acid, and 0.1 (w/v) Coomassie-Brilliant-blue R250. The gel was destained (till the blue protein bands appeared against a clear background) with 30% (v/v) methanol, 10% (v/v) acetic acid and dried overnight between two gel-dry-films (Promega) or placed on top of two sheets of Whatman paper, covered with plastic wrap and dried for 1-2 h in a gel dryer at 80°C.

2.5.7. Transfer of proteins to nitrocellulose/PVDF membrane (Western-Blot)

Proteins separated via SDS-PAGE were transferred onto nitrocellulose membrane using the semi-dry blotting method (Towbin, 1979; Kyhse-Anderson, 1984). The gel, the membrane, and four sheets of Whatman filter paper (3MM) were incubated in transfer buffer (20 mM Tris, 150 mM glycine, 20% (v/v) methanol, 0.08% (w/v) SDS). Two sheets of filter paper were placed on the anode electrode followed by the membrane and the gel. This was covered with other two filter papers and with the cathode electrode. The transfer was performed at 1 mA/cm^2 for 1.5 h. To verify transfer efficiency, the nitrocellulose membranes were reversibly stained with Ponceau S solution (0.2% (w/v) Ponceau S in 3% (w/v) TCA).

Proteins separated via BNGE were transferred onto a PVDF membrane using the semi-dry blotting method described above. Before blotting, the PVDF membrane was activated for 5 min in methanol and 5 min in transfer buffer. After electrophoresis the PVDF membrane was reversibly stained with Coomassie-Brilliant-blue R solution (30% (v/v) methanol, 10% (v/v) acetic acid, 0.1 (w/v) Coomassie-Brilliant-blue R250) for 2 min in order to visualize the marker proteins.

For destaining, a solution of 30% (v/v) methanol and 10% (v/v) acetic acid was used. After completely removing the methanol by washing with TBS buffer (9 g/l NaCl, 10 mM Tris/HCl, pH 7.4) the membrane was immunodecorated or the radioactive material was visualized by autoradiography.

2.5.8. Protein quantification by autoradiography/densitometry and phosphorimaging

Dry membranes containing radiolabelled proteins were exposed to Röntgen films (Autoradiography). The films were scanned and the intensity of bands of interest was quantified (Densitometry). Alternatively, the nitrocellulose membranes were exposed to Phosphroimaging plates and the intensity of the bands was determined with the Phosphorimager (Fuji BAS 1500).

2.5.9. MPP protection assay

Chemical amounts of mitochondrial precursor proteins were incubated in MPP-buffer (50 mM KOAc, 20 mM Tris/HCl, 0.1% digitonin, 1 mM DTT, 2 mM $MnCl_2$, pH 7.4), alone or in the presence of either purified TOM complex or BSA, for 30 min at various temperatures. The recombinant MPP was added and incubation was continued for 8 min at 25ºC. MPP enzymatic activity was inhibited by adding 4 mM EDTA. The proteins contained in the reaction mixture were precipitated with TCA and analyzed by SDS-PAGE.

2.5.10. Immunoblotting

Protein bands immobilized on the nitrocellulose or PVDF membranes could be visualized via decoration with specific antibodies. The non-specific protein binding sites were blocked during membrane incubation with 5% (w/v) skim milk in TBS buffer at RT for 1 h. The immunodecoration was done for 1 h at RT or longer at 4°C, with specific antiserum (1:200 to 1:1000 dilutions in milk/TBS). After washing the membrane 3 times (each 5-10 min), once with TBS, then TBS with 0.05% (v/v) Triton X-100 and again with TBS, it was incubated for 1 h with peroxidase coupled to goat anti-rabbit-IgG or anti-mouse-IgG (diluted 1:10 000 in milk/TBS). The membrane was washed again like described above and treated with Luminol reagents: 2.5 mM aminophalhydrazide and 0.4 mM p-cumaric acid in 0.1 M Tris/HCl pH 8.5, dilution 1:1 with 0.018% (v/v) H_2O_2 in 0.1 M Tris/HCl, pH 8.5. The luminescence reaction was detected with Röntgen films (Fuji NewRX).

3. RESULTS

3.1. *Recognition of preproteins by the isolated TOM complex of mitochondria*

The TOM complex, the protein translocase of the mitochondrial outer membrane, mediates targeting and membrane translocation of the nuclear-encoded preproteins. To better understand how precursor proteins are recognized by mitochondria and translocated across the outer membrane, the interactions of mitochondrial precursor proteins with the TOM complex were analyzed. Two forms of TOM complex were isolated. In the presence of digitonin, a mild detergent, the holo complex containing Tom40, Tom22, Tom5, Tom6, Tom7 and the preprotein receptor components Tom20 and Tom70 could be isolated (Künkele *et al.,* 1998). When the isolation was performed in the presence of a stronger detergent, dodecyl maltoside (DDM) the Tom20 and Tom70 dissociated from the complex, resulting in isolation of the core complex (Ahting *et al.,* 1999). Chemical amounts of recombinant mitochondrial precursors were purified and used in these experiments to eliminate the involvement of chaperones and presequence binding factors usually present in reticulocyte lysate.

3.1.1. Isolated TOM complex is able to bind and partially translocate the preproteins

To study binding of preproteins to isolated TOM holo complex, the sedimentation behavior on sucrose gradients of the purified TOM holo complex, the precursor protein pSu9(1-69)-DHFR (fusion protein consisting of the presequence of Subunit 9 of the Fo-ATPase (amino acid residues 1-69) fused to mouse dihydrofolate reductase (DHFR)), and the adduct between them were analyzed. Free precursor was present at the top of the gradient while the TOM complex was found towards the bottom of the gradient (Fig. 4, upper panel). Incubation of the precursor protein with the TOM complex prior to centrifugation resulted in the co-migration of these components (Fig. 4, lower panel). The specificity of binding was demonstrated in the following experiments: i) upon incubation of excess precursor with the TOM holo complex a major part of it remained unbound (Fig. 4D); thus, the binding sites on the TOM complex could be saturated, ii) DHFR (without a presequence) did not bind to the TOM complex (data not shown), iii) unrelated proteins, whether folded like soybean trypsin inhibitor or unfolded like reduced carboxymethylated lactalbumin (RCMLA), did not bind to the TOM complex (data not shown). Thus, the binding observed was dependent on the mitochondrial targeting signal and did not occur to unrelated unfolded domains. Hence, soluble TOM holo complex can bind precursor proteins in a specific manner. Although some lipid molecules are attached to the purified

TOM holo complex, an intact bilayer structure appears not to be necessary for efficient binding of preproteins to the TOM complex.

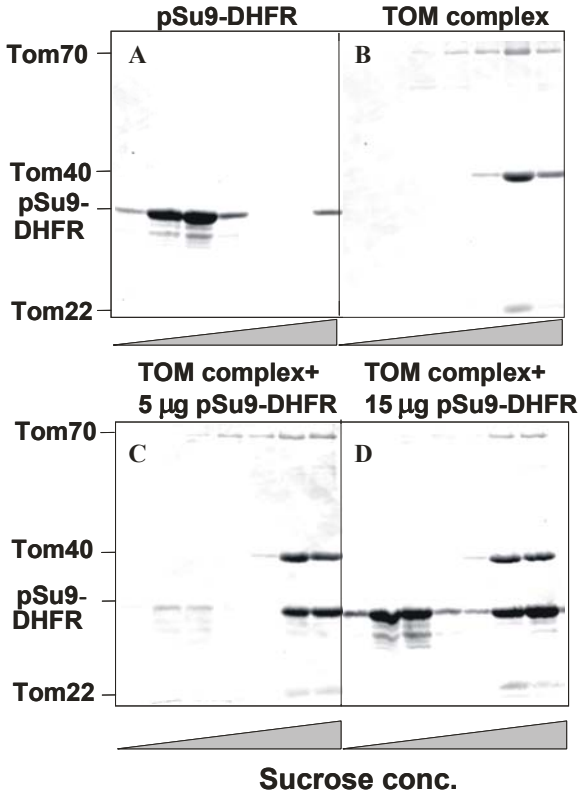

Fig. 4. Analysis of binding of mitochondrial precursor protein to isolated soluble TOM holo complex by sucrose gradient centrifugation. TOM holo complex and pSu9(1-69)-DHFR were incubated either separately (A, B) or together (C, D). In each case, samples were kept for 20 min at 4°C. The amount of TOM holo complex was 24 µg in each sample, and the amounts of pSu9(1-69)-DHFR were 10 µg in A, 5 µg in C and 15 µg in D. After 16 h centrifugation, at 140,000xg on a 0% to 35% sucrose gradient, fractions (100 µl) were collected, TCA was added, and precipitated proteins were analyzed by SDS-PAGE and Coomassie staining.

Can the presequence not only be bound to the isolated complex but also be transferred into the import channel? To answer this question an assay was developed to determine whether binding to the TOM complex can protect a precursor against cleavage by added mitochondrial processing peptidase (MPP). The preproteins tested were pSu9(1-69)-DHFR and pSu9(1-45)-DHFR (amino acid residues 1-45 or 1-69 of Su9 fused to mouse DHFR). Incubation of precursor proteins with MPP in the absence of TOM complex or in the presence of BSA as a control resulted in complete or nearly complete removal of the presequence part of the tested preproteins. In contrast, upon incubation of the preproteins with the TOM holo complex, a major part, if not all, of the precursor molecules were not cleaved by added MPP (Fig. 5A).

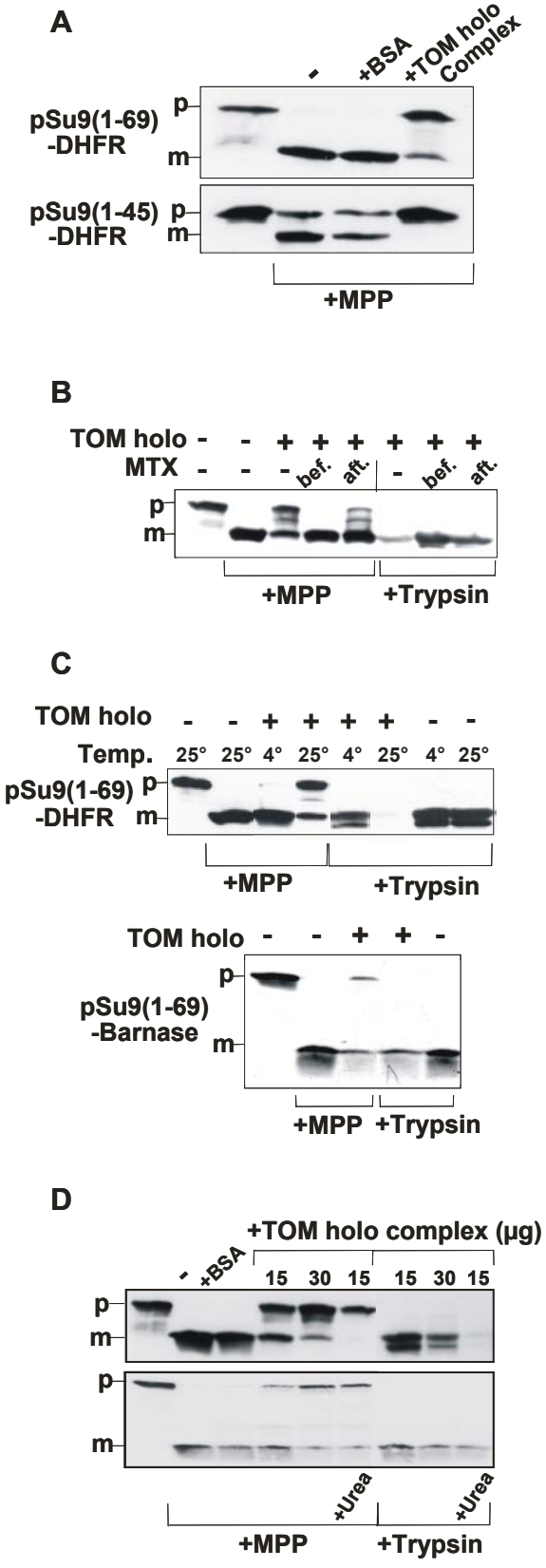

Fig. 5. The TOM complex mediates translocation of the presequence to a site inaccessible to MPP; the translocation requires unfolding of mature domain. (A) The isolated TOM holo complex can translocate the presequence of preproteins to a site inaccessible to MPP. Precursor proteins, pSu9(1-69)-DHFR and pSu9(1-45)-DHFR (3 µg, each), were incubated with 15 µg TOM holo complex (or 15 µg BSA as a control) for 30 min at 25°C. MPP (2 µg) was added for 8 min at 25°C, followed by addition of 4 mM EDTA to stop MPP activity. Proteins were analyzed by SDS-PAGE, blotted and immunodecorated with antibodies against mouse DHFR. **(B)** Stabilization of the mature part influences the protection against MPP. pSu9(1-69)-DHFR (3 µg) was incubated with or without TOM complex (60 µg) for 30 min at 25°C. MTX (1 µM) was present in this incubation mixture (bef.) or was added after incubation for 10 min at 0°C (aft.). Samples containing TOM complex were then split and to one half MPP (15 µg/ml) was added for 8 min at 25°C while to the other trypsin (20 µg/ml) was added for 15 min at 0°C. Trypsin was inhibited with 1 mM PMSF. Further treatment was as above. **(C)** Unfolding of the mature part is required to achieve of protection against MPP. pSu9(1-69)-DHFR (3 µg) (upper panel) was incubated in the absence or presence of TOM complex (60 µg) for 30 min at 4°C or 10 min at 25°C. MPP and trypsin treatment was as above. pSu9(1-69)-barnase (3 µg) (lower panel) was incubated in the absence or presence of TOM complex (30 µg) for 30 min at 25°C. MPP and trypsin treatment was as above. Proteins were analyzed as above and immunodecorated with antibodies against barnase. **(D)** Unfolding of preproteins before their interaction with the TOM complex increases their extent of protection against MPP. Preproteins (pSu9(1-69)-DHFR upper panel and pSu9(1-69)-barnase lower panel, each 3 µg) were incubated in the absence or presence of the indicated amounts of TOM holo complex for 30 min at 25°C. Where indicated (+Urea) the preprotein was incubated first in 8 M urea for 30 min at 25°C and then diluted into the binding reaction. Further treatment was as above. p and m, precursor and mature forms of preproteins.

3.1.2. The partial translocation of the precursor is dependent on unfolding/stability of the mature part

To check whether the observed protection is dependent on the unfolding of the DHFR domain, binding and processing were performed in the presence or absence of methrotrexate (MTX), a ligand that stabilizes the folded conformation of the DHFR domain. If MTX was added before incubation of the precurosor with the TOM complex to allow stabilization of the DHFR domain, the precursor was not protected against MPP (Fig. 5B). Thus, the presequence can penetrate the channel and in this way to become inaccessible to MPP only when the DHFR domain is unfolded. This unfolding was verified by trypsin degradation of the DHFR domain. If the MTX was added after binding of the precursor to the TOM complex, an intermediate situation was observed where only a smaller fraction of the precursor was protected against MPP and unfolded (Fig. 5B). To check if TOM complex can unfold preproteins or can only stabilize their unfolded comformation, the unfolding reaction was studied at various temperatures. Partial translocation and unfolding were observed when the initial incubation was at 25°C, but not at 4°C. Thus, the isolated TOM complex retains its ability to stabilize the unfolded conformation of the DHFR domain at 25°C *in situ*, i.e. in intact mitochondria and outer membrane vesicles (Mayer *et al.,* 1995c; Rapaport *et al.,* 1998b), (Fig. 5C, upper panel).

To check if the TOM complex can stabilize the unfolded conformation of a more stable domain than DHFR, the binding and protection of pSu9(1-69)-DHFR and pSu9(1-69)-barnase were compared. Barnase is a small ribonuclease of 110 amino acid residues; it was reported that the spontaneous unfolding of DHFR at 30°C is 30 fold faster than that of barnase, (Matouschek *et al.,* 1997). At 25°C, a fraction of pSu9(1-69)-barnase molecules were partially translocated into the channel and unfolded, as measured by protection against MPP treatment and trypsin treatment, respectively. In agreement with its higher stability, the precursor containing the barnase domain was protected less effectively than the DHFR-containing protein (Fig. 5C, lower panel). Thus, it can be concluded that the unfolding process is indeed the limiting step in the translocation process. To verify this point both precursor proteins were first denatured in 8 M urea and then incubated with the TOM complex. The unfolded precursors (as tested by trypsin degradation) were protected against MPP to a greater extent than the native precursors; no processed protein was observed after denaturation (Fig. 5D). These results confirm that transfer of the presequence to a site protected from MPP depends on unfolding.

3.1.3. The receptors are not essential for partial translocation and unfolding

To investigate if the preprotein receptors are essential for partial translocation and unfolding of the preproteins, the TOM holo complex and OMV purified from *N. crassa* mitochondria were treated with low concentration of proteinase K, which resulted in the cleavage of Tom70 and Tom22 and most of Tom6 (Fig. 6A and 6B, respectively). Under these conditions the full-length Tom20 was not detected in the treated complex, while residual amounts of characteristic proteolytic fragments were still observed (Fig. 6A). Tom40 stayed intact under these conditions. Both treated complex and OMV were also able to provide protection against MPP (Fig. 6A and 6B, respectively). From this experiment it was concluded that Tom40 plays the central role in the capacity of the soluble complex to translocate the presequence part into the translocation pore.

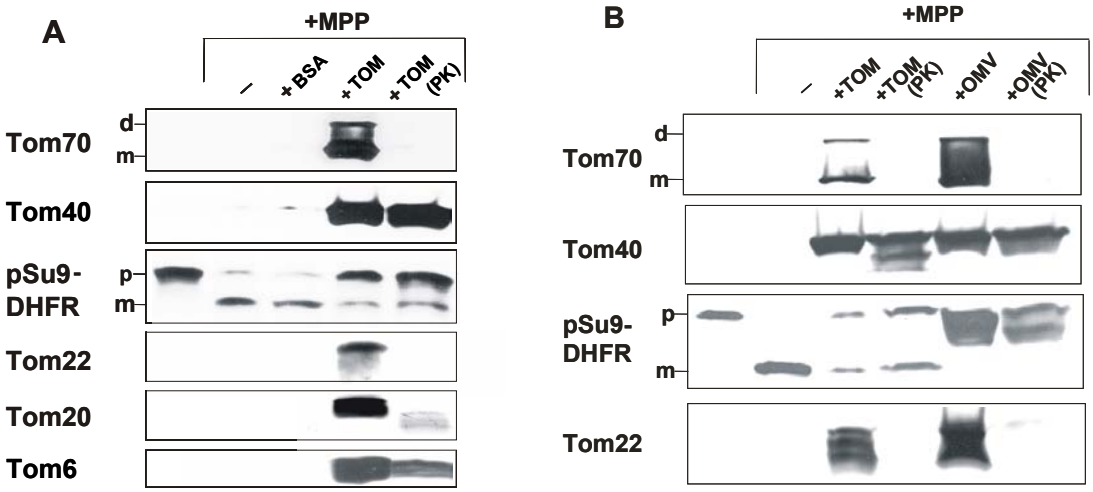

Fig. 6. Receptor proteins are not required for translocation to a site protected against MPP. **(A)** pSu9(1-45)-DHFR (3 µg) was incubated for 30 min at 4°C with 15 µg of either TOM holo complex or TOM holo complex treated with proteinase K, or with BSA as a control. MPP was then added for 8 min at 25°C. Proteins were analyzed by SDS-PAGE; blotting and immunodecoration with antibodies against DHFR or Tom components were performed. The bands corresponding to the monomer (m) and dimer (d) forms of Tom70 and of precursor and mature forms of the precursor protein (p and m, respectively) are indicated. **(B)** pSu9(1-69)-DHFR (3 µg) was incubated for 30 min at 4°C with 15 µg of either TOM holo complex or TOM holo complex treated with proteinase K, or incubated for 20 min at 25°C with 200 µg of either OMV or OMV treated with proteinase K. Further treatment was as above.

3.1.4. Lipids are required for the proper function of the TOM complex

Based on the results presented in Fig. 6 it is expected that the TOM core complex is also able to unfold and partially translocate precursor proteins. Surprisingly, after incubation with the TOM core complex, the precursor could not be protected against MPP (Fig. 7A).

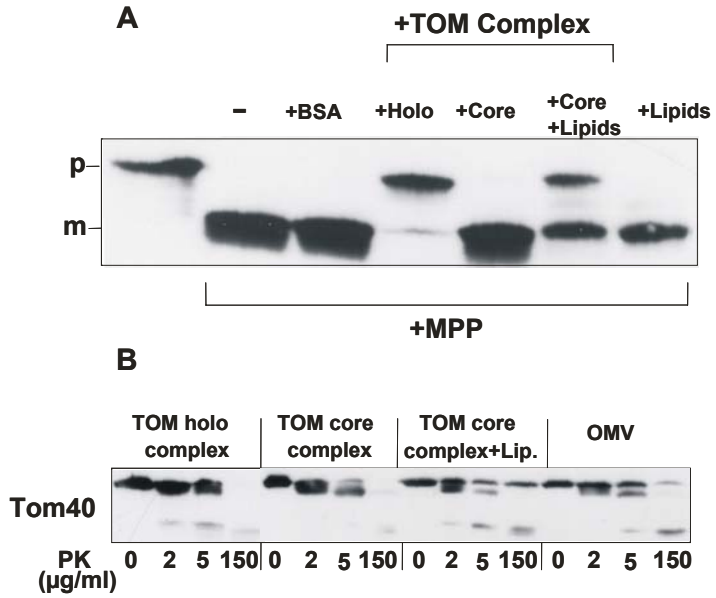

Fig. 7. Addition of phospholipids restores the translocation activity of the TOM core complex; phospholipids affect the conformation of the TOM core complex. pSu9(1-69)-DHFR (3 µg) was incubated in the absence or presence of TOM complex (30 µg) for 30 min at 25°C. In the indicated sample phospholipids (200 µg) were incubated with TOM core complex for 1 h at 4°C before addition of the preprotein. In control sample phospholipids without TOM complex were incubated with the preprotein. MPP was then added to the indicated samples for 8 min at 25°C. p and m, precursor and mature forms of preprotein. **(B)** Phospholipids affect the conformation of the TOM core complex**.** TOM holo complex (10 µg), TOM core complex (10 µg) with or without added phospholipids (67 µg), or OMV (80 µg) were treated with the indicated amounts of proteinase K (PK) for 15 min at 0°C. Then PK was inhibited by PMSF, and proteins were analyzed by SDS-PAGE, blotting and immunodecoration with antibodies against Tom40 were performed.

The core complex was purified after solubilization of the mitochondria in buffer containing DDM as a detergent instead of digitonin, which was used for the purification of the TOM holo complex. We found that the phospholipid content of the TOM core complex is much lower than that of the TOM holo complex. The amount of phospholipid present was 4 mol/mol TOM complex in the DDM-solubilized complex and 168 mol/mol TOM complex in the digitonin-solubilized complex. The lower activity of the TOM core complex may therefore result from the lower levels of structural lipid molecules. Phospholipids have been shown to play an important role in protein translocation (van Voorst and Kruijff, 2000). Such lipid molecules could be required for the proper function of the complex, similar to the finding that non-bilayer lipids stimulate the activity of the reconstituted bacterial protein translocase (van der Does *et al.,* 2000). Lipids from the mitochondrial outer membrane were extracted and added to the TOM core complex in

order to check if higher lipid content restores the activity of this complex. Indeed, the precursor incubated with the TOM core complex supplied with lipids was partially protected against MPP (Fig. 7A).

Beside restoration of the protection activity, the higher content of lipids also induces a change in the pattern of the proteolytic fragments of Tom40. The pattern of the TOM core complex supplied with lipids resembled more closely that of the native complex in the outer membrane vesicles. Hence, lipids induce a more "native-like" conformation of the TOM complex and thus cause the complex to support translocation, which results in protection against MPP (Fig. 7B).

Taken together, purified precursors were found to bind efficiently to isolated TOM complex in the absence of chaperones and lipids in a bilayer structure. In addition to recognition, the purified complex is able to transfer the presequence part of the precursors from an exposed location at the complex to a location inaccessible to added MPP, most likely into the translocation pore.

3.2. *Recognition of BCS1 precursor by the TOM complex*

BCS1 protein is a mitochondrial inner membrane protein and a member of the highly conserved ATPases, the AAA protein family (Beyer, 1997; Patel and Latterich, 1998). The BCS1 protein is anchored in the mitochondrial inner membrane via a single transmembrane domain. A short N-terminal tail is exposed into the intermembrane space, whilst the bulk of the protein is in the matrix (in a N_{out}-C_{in} orientation). The transmembrane anchor is followed by a short positively charged segment (Fig.8) (Fölsch *et al.,* 1996).

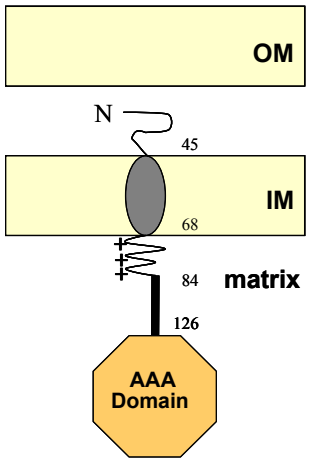

Fig. 8. Topology and internal targeting signal segments of BCS1. Four sequence elements could be identified in BCS1: (i) the N-terminal residues 1-44, (ii) a transmembrane domain at amino acids 45-68, (iii) a presequence-like helix (residues 69-83), and (iv) an import auxiliary region at residues 84-126.

3.2.1. BCS1 interacts with the outer mitochondrial membrane via both electrostatic and hydrophobic interactions

To study the interactions of BCS1 precursor with the outer mitochondrial membrane, a hybrid protein composed of the first 126 amino acid residues of BCS1 fused to DHFR (BCS1(1-126)-DHFR) was synthesized *in vitro* and incubated with OMV isolated from *N. crassa* mitochondria. This fusion protein was shown previously to be correctly sorted to the inner membrane and imported into mitochondria with a rate and efficiency similar to that of authentic BCS1 protein (Fölsch *et al.,* 1996).

The binding properties of this precursor protein were compared to those of a matrix destined precursor, pSu9-DHFR. Whereas the binding of the matrix-destined precursor, pSu9-DHFR at low temperature was completely salt-sensitive, a significant portion of BCS1(1-126)-DHFR molecules remained bound to OMV after a treatment with 200 mM salt (Fig. 9A). It had been already shown that Su9-DHFR, when arrested at the *cis* site (0°C, +MTX), was loosely associated with the TOM complex, even when the incubation was performed in the presence of low salt concentration (Rapaport *et al.,* 1998b). Therefore, hydrophobic interactions seem to play a more important role in mediating binding of BCS1(1-126)-DHFR under these conditions. About half of the salt-resistant BCS1(1-126)-DHFR molecules contained a folded DHFR domain (Fig. 9A). Hence, these hydrophobic interactions are probably mediated by the BCS1 moiety of the fusion protein and not by the folded DHFR domain. When the binding was allowed to occur at 25°C, unfolding of the DHFR domain and resistance to treatment with high salt buffer were observed with both precursors (data not shown).

3.2.2. The isolated TOM complex can bind the precursor of BCS1

To check if the interactions of BCS1 with the outer membrane are indeed mediated by the TOM complex, immunoprecipitation experiments were performed. The precursors of BCS1wt and BCS1(1-126)-DHFR were incubated with OMV; the TOM complex was isolated by immunoprecipitation, and analyzed for bound preprotein. A substantial fraction of both precursors were co-immunoprecipitated with the TOM complex after preprotein binding at 25°C and wash with high salt buffer (Fig. 9B). Lower amounts of precursors were associated with the TOM complex when the binding was analyzed at 0°C. The relatively stable binding of BCS1 (and BCS1(1-126)-DHFR) to the TOM complex at 0°C (see also Fig. 9A) differs from the loose binding of the matrix destined precursor, pSu9-DHFR under similar conditions (Rapaport *et al.,* 1998b).

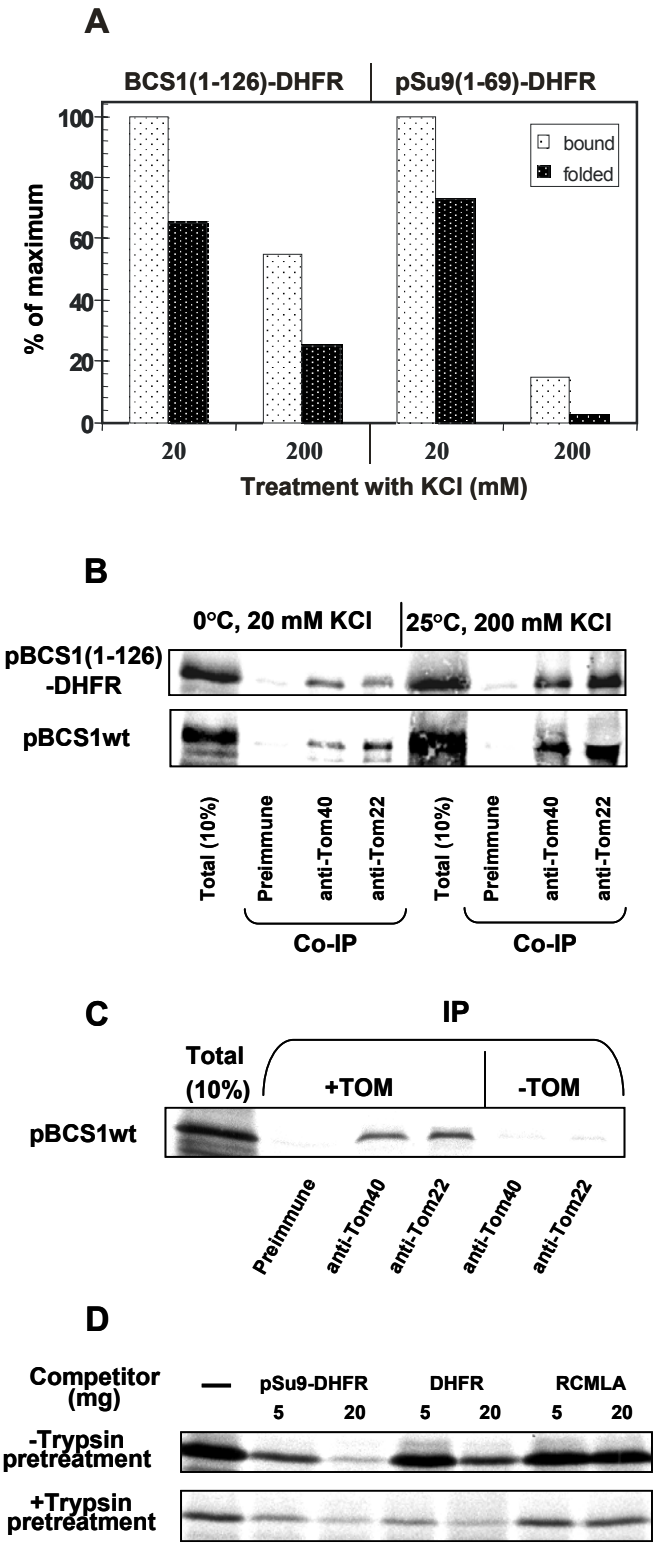

Fig. 9. Interaction of the targeting signal of BCS1 with the TOM complex (A) BCS1(1-126)-DHFR and pSu9(1-69)-DHFR were incubated with *N. crassa* OMV for 20 min at 0°C in the presence of MTX/NADPH. OMV were then treated with buffer containing either 20 or 200 mM KCl. The OMV were reisolated, resuspended in buffer, and the suspensions halved. One half was treated with trypsin at 0°C ("folded" material), while the second half was kept at 0°C ("bound"). Proteins were then analyzed by SDS-PAGE and phosphorimaging. The amount of protein bound at 20 mM salt was set to 100%. **(B)** Preprotein bound to OMV can be co-immunoprecipitated with components of the TOM complex. Radiolabelled BCS1 and BCS1(1-126)-DHFR were incubated with OMV at 0°C in the presence of MTX/NADPH and 20 mM KCl, or at 25°C in the presence of 200 mM KCl and absence of MTX/NADPH. After the binding reaction OMV were reisolated and resuspended in SEM buffer. Immunoprecipitation was performed with antibodies raised against Tom22, Tom40 or with preimmune serum. To control the binding, an aliquot was removed before the co-immunoprecipitation and precipitated with TCA (Total). **(C)** Precursor of BCS1 interacts with purified TOM complex. Radiolabelled precursor of BCS1 was incubated for 20 min at 25°C with purified TOM core complex. The reaction mixture was split as described in part (B) and immunoprecipitation was performed. To exclude unspecific interactions, immunoprecipitation was also performed in the absence of the TOM complex ("-TOM"). **(D)** A matrix-destined precursor can compete out the precursor of BCS1(1-126)-DHFR. Radiolabelled precursor of BCS1(1-126)-DHFR was incubated for 20 min at 25°C with either mitochondria ("-") or with mitochondria preincubated with the indicated amounts of proteins for 2 min on ice. The mitochondria were either intact (upper panel) or pretreated with trypsin before incubation with proteins (lower panel). At the end of the import reactions mitochondria were treated with proteinase K, washed, reisolated, and analyzed by SDS-PAGE.

In the first part of my work I observed that interaction with a bilayer structure is not a prerequisite for interaction of matrix-targeting-signal preproteins with the TOM holo complex (Fig. 4 and 5). To check if this is valid also for proteins with an internal targeting signal, like BCS1, radiolabelled BCS1 was incubated with the purified TOM core complex and the ability of antibodies against Tom components to precipitate it, was tested (Fig. 9C). BCS1 could be precipitated with antibodies against Tom40 and Tom22, suggesting that the BCS1 precursor is interacting with the TOM complex during its import into mitochondria. Apparently, neither lipids in bilayer form nor other proteins in the outer membrane are required for the recognition of the BCS1 precursor by the TOM complex.

3.2.3. The import pathway of the BCS1 precursor

Does the BCS1 precursor use the general import pathway? To address this question, the capacity of a protein destined to the matrix, pSu9-DHFR, to compete out the import of the BCS1 precursor was tested. This approach was used before to demonstrate that outer membrane proteins use the TOM complex for their insertion into the outer membrane (Rapaport and Neupert, 1999; Dembowski *et al.,* 2001; Krimmer *et al.,* 2001). The addition of chemical amounts of pSu9-DHFR during import of radiolabelled BCS1(1-126)-DHFR to yeast mitochondria resulted in a strong reduction of import of the radiolabelled precursor (Fig. 9D, upper panel). In contrast, addition of unrelated proteins like DHFR alone (without presequence) or RCMLA had only a very minor effect on the import of BCS1(1-126)-DHFR. The slight inhibitory effect of DHFR alone may result from the cryptic mitochondrial targeting signal within this protein (Hurt *et al.,* 1987), or from hydrophobic interactions of the TOM complex with unfolded DHFR molecules. Hence, BCS1 and precursors that use the general import pathway share at least one common step in their translocation pathway.

To find out if the inhibitory effect is due solely to competition for binding sites on the receptors of the outer membrane, or also for the import pore, mitochondria were treated with trypsin to remove the surface receptors and incubated with radiolabelled BCS1(1-126)-DHFR. Under these conditions precursors that depend on receptors for import have been shown to enter mitochondria at a lower rate due to "bypass import" which occurs by their direct interaction with the general import pore (Pfaller *et al.,* 1989). This was also true for BCS1(1-126)-DHFR (Fig. 9D, compare first lanes in upper and lower panels). In the presence of excess unlabelled pSu9-DHFR the level of BCS1(1-126)-DHFR import was strongly reduced (Fig. 9D, lower panel). Thus, the import of BCS1 is dependent on import receptors and on components of the translocation pore.

3.2.4. The receptor proteins Tom70 and Tom20 are involved in the recognition of the BCS1 precursor

To identify the Tom receptor subunits that contribute to the recognition of BCS1, the cytosolic domains of Tom20 and Tom70 were blocked by incubation of mitochondria with specific IgGs. The import of both BCS1(1-126)-DHFR and the control precursor, pSu9(1-69)-DHFR, to mitochondria was strongly reduced upon addition of IgG against either Tom20 or Tom70, but not by IgG purified from preimmune serum (Fig. 10).

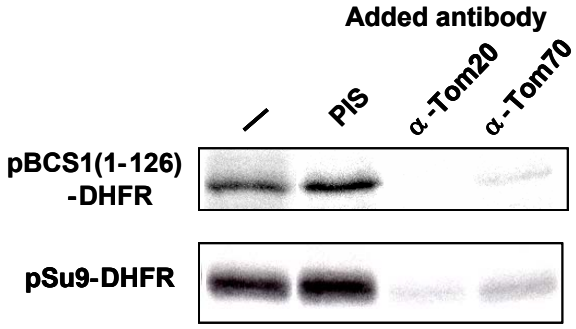

Fig. 10. Precursor of BCS1 interacts with receptor components of the TOM complex. Antibodies against import receptors can inhibit import of BCS1. Mitochondria isolated from *N. crassa* were incubated with IgGs isolated from preimmune serum (PIS), antiserum against Tom 20, or antiserum against Tom 70. Each sample was halved and aliquots were further incubated for 20 min at 25°C with either BCS1(1-126)-DHFR or with pSu9(1-69)-DHFR for control. The import reactions were treated with proteinase K, reisolated and analyzed by SDS-PAGE.

The inhibitory effect of antibodies against Tom70 on the import of pSu9(1-69)-DHFR was unexpected. It may well be that this effect is due to steric hindrance at the TOM complex. The involvement of Tom70 in the binding of BCS1 to mitochondria fits well with earlier observations that Tom70 promotes productive binding and import of pre-cytochrome c_1, another inner membrane protein. Tom70 is known to promote productive binding and import of precursors of inner membrane proteins with internal import signals (Hines *et al.,* 1993; Schlossmann *et al.,* 1994). Therefore it appears that efficient import of BCS1 requires both receptors, Tom20 and Tom70.

To obtain more information on the interactions of the BCS1 precursor with specific Tom components chemical cross-linking experiments were performed. Radiolabelled BCS1 or BCS1(1-126)-DHFR precursors were accumulated as import intermediates in OMV and the homobifunctional cross-linking reagents 1,5-difluoro-2,4-dinitrobenzene (DFDNB) or disuccinimidyl suberate (DSS), respectively were added. Upon addition of DFDNB cross-linking adducts of BCS1 to Tom20, Tom22, and Tom40 were observed (Fig. 11A).

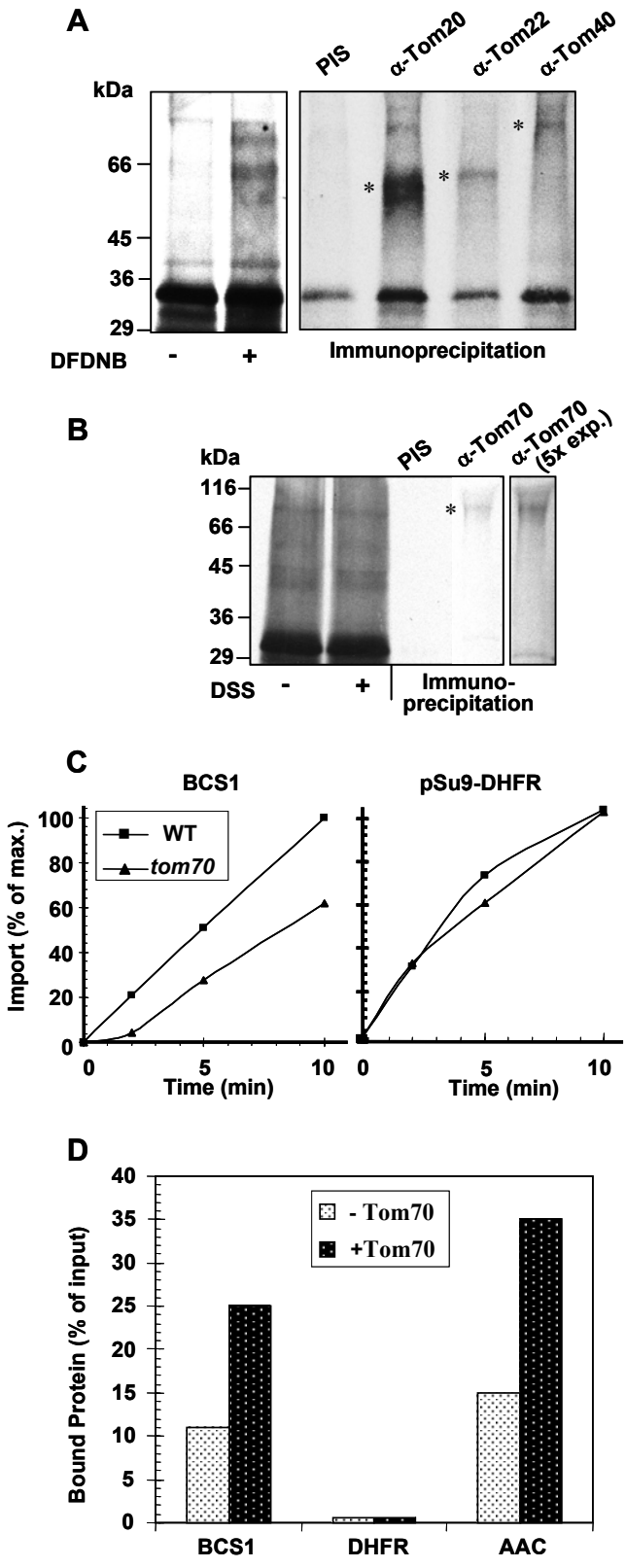

Fig. 11. Precursor of BCS1 interacts with receptor components of the TOM complex. (A) BCS1 is in the vicinity of Tom20, Tom22, and Tom40 on its insertion pathway. Radiolabelled BCS1(1-126)-DHFR precursor was incubated with isolated OMV for 30 min at 0°C. OMV were reisolated and resuspended in SEM buffer. One aliquot was left on ice ("-DFDNB") while the chemical cross-linker DFDNB was added to the others for 40 min on ice. Aliquots were subjected to immunoprecipitation with antibodies against Tom20, Tom22, Tom40 or with preimmune serum (PIS). Cross-link adducts of BCS1 to Tom proteins are labelled by an asterisk. **(B)** BCS1 is in the vicinity of Tom70 on its insertion pathway. Radiolabelled BCS1(1-126)-DHFR precursor was incubated with isolated OMV for 2 min at 0°C. OMV were split and one aliquot was left on ice ("-DSS") while the chemical cross-linker DSS was added to the others for 40 min on ice. Aliquots were subjected to immunoprecipitation with antibodies against Tom70 or with preimmune serum (PIS). Cross-link adduct of BCS1 to Tom70 is labelled by an asterisk. A longer exposure of the immunoprecipitation with Tom70 is presented for clarity. **(C)** The *tom70* null mutation affects import of BCS1. Radiolabelled precursors of BCS1 and pSu9-DHFR were incubated at 15°C for the indicated time periods with mitochondria from either *tom70* null mutant (*tom70*) or its wild type parent (WT). At the end of the import reactions proteinase K was added, mitochondria were reisolated, and analyzed by SDS-PAGE. The protease protected bands of BCS1 and mature Su9-DHFR were quantified. Black squares: wild-type mitochondria; black triangles; mitochondria from the *tom70* null mutant. **(D)** Binding of mitochondrial preproteins to purified Tom70 cytosolic domain. The purified cytosolic domain of Tom70 was bound to Ni-NTA column. Then radiolabelled preproteins were incubated with the bound protein for 30 min at 4°C. After a washing step, the bound proteins were eluted with sample buffer and analyzed by SDS-PAGE. The total amount of each preprotein added was set to 100%.

The Tom20-containing adduct was prominent probably because under the conditions of the binding assay (0°C) association of BCS1 with the receptors rather than with pore components is favored. Using DSS a cross-linking adduct of BCS1(1-126)-DHFR with Tom70 was detected (Fig. 11B). Hence, this experiment supports the idea that both receptors, Tom20 and Tom70, are in the vicinity of the BCS1 precursor in transit.

To demonstrate a functional role of Tom70 in the import of BCS1, radiolabelled BCS1 (or pSu9-DHFR as control) was imported into mitochondria isolated from either a wild type strain or from a strain lacking Tom70. Whereas the control precursor (pSu9-DHFR) was imported into the *Δtom70* or the wild type mitochondria with a similar efficiency, the extent of import of BCS1 into the variant organelles was reduced to about half (Fig. 11C). This reduction is similar to that reported for a well-known substrate of Tom70, AAC, (Hines *et al.,* 1993).

The capacity of Tom70 to bind the precursor of BCS1 was further tested. The His-tagged version of the cytosolic domain of Tom70 was cloned into an over-expressing vector in *E. coli* (Young *et al.,* 2003). The soluble domain was purified and was bound to Ni-NTA beads. The recombinant protein could bind specifically radiolabelled BCS1 precursor. As controls, Tom70 interacted with the known substrate, AAC while the cytosolic protein, DHFR, was bound only at background levels (Fig. 11D). Both BCS1 and AAC precursors contain hydrophobic stretches and hence have some unspecific binding to the Ni-NTA beads (Fig. 11D). The involvement of Tom70 in the recognition of the BCS1 precursor is supported by experiments with a construct composed of amino acid residues 66-86 of BCS1 fused to DHFR. As presented below, this construct can be efficiently cross-linked to Tom70 and immunoprecipitated by antibodies against the latter protein (Fig. 15C and 15D). Taken together, these results clearly demonstrate that on its import pathway BCS1 is recognized by the import receptor Tom70.

3.2.5. The import signal of BCS1

The region of amino acid residues 1-126 of BCS1 contains two putative structural elements; a hydrophobic stretch (a. a. 45-68) that most likely forms the single transmembrane segment of the protein, and an amphiphilic α-helix (a. a. 69-83) that resembles a presequence. Previous work has shown that both elements are essential for the proper import and sorting of the protein (Fölsch *et al.,* 1996).

To determine the elements in BCS1 which bind to the receptor components of the TOM complex peptide scans were performed (Fig. 12).

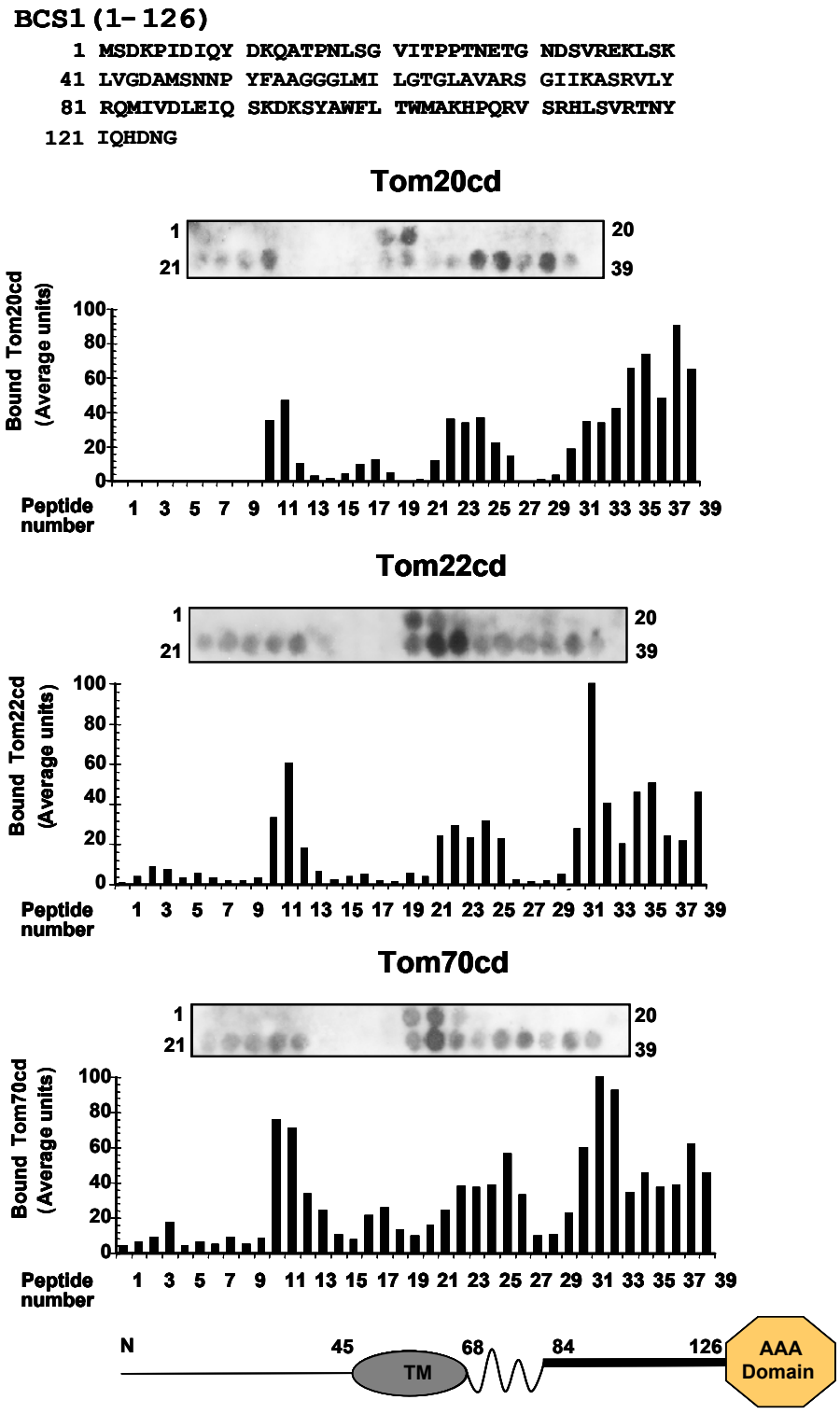

Fig. 12. Screening of a peptide library with soluble receptor domains. Cytosolic domains of the indicated Tom components (150 nM) were incubated with a peptide library on a cellulose membrane covering amino acid residues 1-126 of BCS1 (length of peptides 13 residues, overlap 10 residues). The bound proteins were blotted to PVDF membranes and decorated with the corresponding antibody. The labeling indicates the number of the peptide in the beginning and the end of each row. Binding was quantified by scanning densitometry from three independent experiments. The various domains of BCS1 are displayed below the corresponding peptides. TM, transmembrane domain; H, putative presequence-like helix.

The peptide library consisted of 13mers over-lapping by 10 residues and covered residues 1-126 of BCS1. The peptides were attached via their C-terminal residues to a cellulose membrane (Kramer *et al.,* 1998). The soluble domains of the Tom receptor subunits, Tom20, Tom22 and Tom70 were purified as described (Brix *et al.,* 1997), and incubated with the cellulose membrane. Bound proteins were transferred to a PVDF membrane which was then immunodecorated with antibodies against the various Tom subunits.

All three receptors bind very weakly or not at all to peptides covering the first 30 amino acid residues, and the region of the transmembrane domain (Fig. 12). Relatively strong interaction was observed with two peptides covering residues 31-46. Both peptides contain three positively charged residues in positions 35, 37 and 40 which could be involved in this binding. Moderate to high binding of all three receptors was observed to peptides covering the presequence-like helix at residues 68-83 (peptides 23-25, Fig. 12).

Binding to the three receptors was strongest at the region of amino acid residues 92-126. While Tom20 displayed highest affinity to a stretch between amino acids 103 to 126 which contains 4 positively charged residues, Tom22 and Tom70 had a clear preference for residues 94-106 which comprise lysine residues at both termini (Fig. 12). These results suggest that several segments of BCS1, but not the transmembrane domain can be bound by the three Tom receptor proteins. Residues 92-126 of BCS1 seem to play an important role in the recognition of the protein by the Tom receptor subunits.

Interaction of the transmembrane domain of the BCS1 with the TOM complex

The overall import of BCS1 into the mitochondrial inner membrane requires the transmembrane domain of the protein. On the other hand, a BCS1 construct lacking the transmembrane segment was observed to bind to the outer surface of mitochondria *in vitro* where it was completely exposed to added protease (Fölsch *et al.,* 1996).

To test whether the transmembrane segment has a major role in the suggested hydrophobic interaction of BCS1 precursor with the TOM complex, the binding of a hybrid precursor protein lacking this domain, BCS1(1-126)ΔTM-DHFR to OMV was tested. The stability of its binding was reduced in the presence of higher salt concentrations (Fig. 13A). However, even under high salt its association with the TOM complex was more stable than that of the matrix destined precursor, pSu9-DHFR (Fig. 13A). Thus, in addition to the transmembrane domain further elements of BCS1 contribute to its strong interaction with the TOM complex.

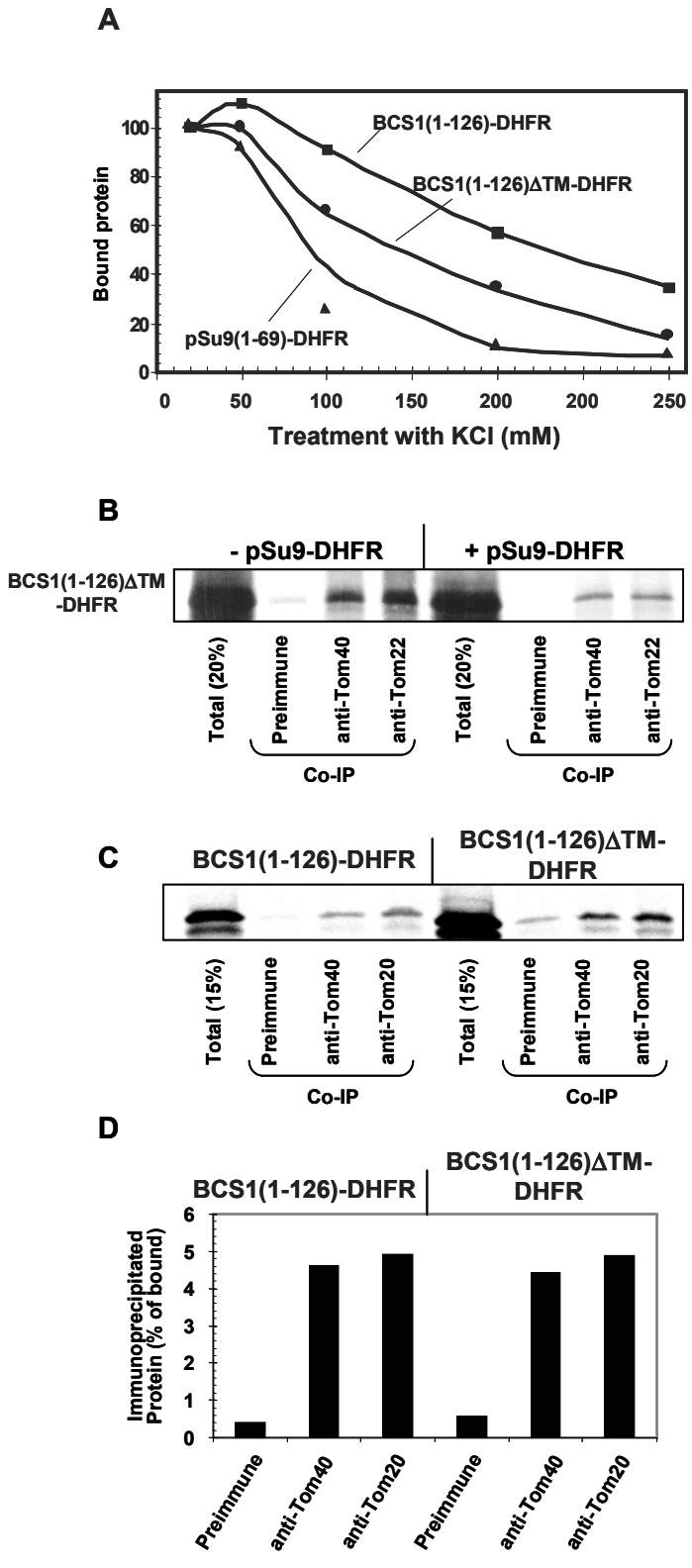

Fig. 13. BCS1 can bind to the TOM complex in the absence of the transmembrane domain. (A) The indicated radiolabelled precursors were incubated with OMV for 20 min at 0°C. Samples were then adjusted to the indicated KCl concentrations. OMV were reisolated, dissolved in sample buffer and analyzed for bound precursor proteins by SDS-PAGE and phosphorimaging. The amount of preprotein bound at 20 mM salt was set as 100%. **(B)** pBCS1(1-126)ΔTM-DHFR was incubated with OMV for 20 min at 25°C in the presence or absence of chemical amounts of pSu9(1-69)-DHFR. The reactions were made 200 mM KCl at 0°C, and OMV were reisolated and resuspended in SEM buffer. Immunoprecipitation was performed with antibodies raised against Tom22, Tom40 or with preimmune serum. To control for binding, an aliquot was removed before the co-immunoprecipitation and precipitated with TCA (Total). **(C)** pBCS1(1-126)-DHFR and pBCS1(1-126)ΔTM-DHFR were incubated with uncoupled mitochondria for 20 min at 25°C. The mitochondria were washed with a buffer containing 50 mM KCl, re-isolated and solubilized in 0.75% digitonin buffer. Further treatment and co-immunoprecipitation were as described in the legend to Fig. 9B. **(D)** The bands corresponding to immunoprecipitated proteins from the experiment described in (C) and two other such experiments were quantified. The average values as percentage of the bound material are presented.

The construct lacking the transmembrane domain was incubated with OMV in the presence or absence of competing chemical amounts of pSu9-DHFR (Fig. 13B). Binding and precipitation by antibodies against Tom components were reduced when the competing precursor was present (Fig. 13B). Apparently, the BCS1 precursor recognizes binding site(s) on the general import pathway even in the absence of the transmembrane domain. Next, a comparative co-immunoprecipitation experiment was performed. Both BCS1(1-126)-DHFR and BCS1(1-126)ΔTM-DHFR were incubated with uncoupled mitochondria and the capacity of antibodies against either Tom20 or Tom40 to precipitated the import intermediates was tested (Fig. 13C). Quantification of three such experiments revealed that the levels of precipitation were very similar for both precursors (Fig. 13D). Together with the results of the peptide binding experiment, these experiments demonstrate that the transmembrane domain is not required for the stable interaction with the TOM complex.

The amino acid sequence of the transmembrane segment of BCS1 is highly conserved among different species (see below, Fig. 20). This segment may have a specific role in the function and/or sorting of BCS1, rather than merely serving as a hydrophobic membrane anchor. To check whether a transmembrane domain of another inner membrane protein would be able to functionally replace the native transmembrane domain of BCS1, a BCS1 variant containing the transmembrane segment of cytochrome c_1, was constructed. Cytochrome c_1 is an inner membrane protein that, like BCS1, has a N_{out}-C_{in} topology (Fig. 14 scheme).

This variant, BCS1-Cytc_1, was not properly imported *in vitro* into the inner membrane upon incubation with isolated mitochondria. Similar to the construct lacking the transmembrane domain, BCS1-Cytc_1 binds to the outer membrane but remains exposed to added protease (Fig. 14A lower panel). The bound BCS1-Cytc_1 was associated with the TOM complex as it could be immunoprecipitated with antibodies against Tom22 and Tom40 (data not shown).

To test whether these *in vitro* results also reflect also the *in vivo* situation, BCS1-Cytc_1 was cloned into a yeast expression vector and transformed into the *BCS1* null strain.

Mitochondria from these transformed cells were isolated and treated with proteinase K. Like in the *in vitro* experiments the protein was exposed on the mitochondrial outer membrane and not properly sorted to the inner membrane (data not shown). A plasmid containing the BCS1-Cyt*c*1 did not rescue the *pet* phenotype resulting from deletion of the chromosomal *bcs1* gene, and the transformed cells did not grow on non-fermentable carbon source (Fig. 14B).

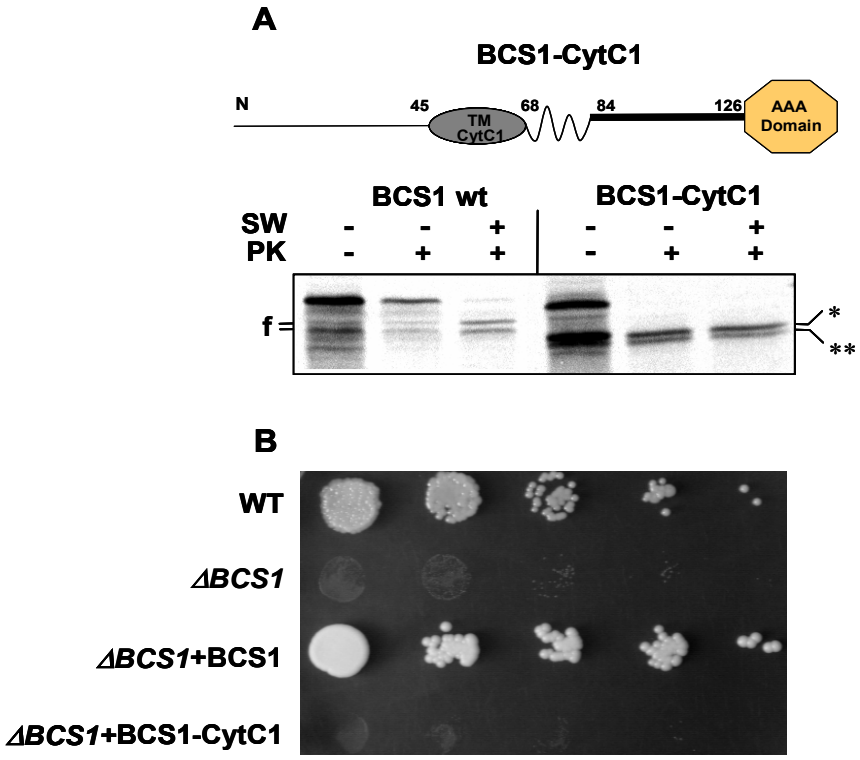

Fig. 14. The transmembrane domain of BCS1 contains specific information for the correct sorting. (A) A precursor containing the transmembrane domain of Cyt*c*1 is not imported properly *in vitro*. Radiolabelled precursors of BCS1 and BCS1-Cyt*c*1 (see scheme) were imported into yeast mitochondria for 20 min at 25°C. Each sample was split into three aliquots and diluted 10 times with either SH buffer (-SW, -PK), SH buffer containing proteinase K (-SW, +PK), or 20 mM Hepes containing proteinase K (+SW, +PK). After further incubation on ice for 30 min the protease was inactivated by PMSF. f, fragments generated by proteinase K treatment of ruptured mitochondria. The product resulting from translation starting at Met68 and its corresponding processed form are indicated by one or two asterix, respectively. **(B)** BCS1-Cyt*c*1 cannot functionally replaced BCS1. *BCS1Δ* cells, their isogenic wild type, and *BCS1Δ* cells transformed with either BCS1 coding sequence or sequence encoding BCS1-Cyt*c*1 were tested by dilutions in 10-fold increments for their ability to grow at 30°C on YPG medium.

Similar results were obtained using a strain where the *bcs1* gene was deleted in a different genetic background (data not shown). Hence, BCS1-Cyt*c*1 associates with the mitochondrial outer membrane but is not imported further. These results suggest that the transmembrane segment of BCS1 cannot be replaced by a hydrophobic transmembrane domain of another inner membrane protein.

Interaction of the presequence-like domain of the BCS1 with the TOM complex

The other structural element in the import signal of BCS1 is an amphipathic helix that similarly to N-terminal mitochondrial targeting sequences is rich in positively charged residues. To check whether this import signal follows the general import pathway, the latter was saturated with chemical amounts of matrix-destined precursor and import of radiolabelled pBCS1Δ65 and of pSu9(1-69)-DHFR as control was analyzed. Import of both precursor proteins was inhibited by the mitochondrial preprotein but not by the unrelated protein RCMLA (Fig. 15A). Hence, pBCS1Δ65 interacts with the TOM complex in a manner similar to matrix-destined precursors.

To test whether the presequence-like segment has the capacity to target a cytosolic protein to the mitochondria, a chimeric precursor protein, BCS1(66-86)-DHFR was constructed. This precursor contains the presequence-like segment (a. a. residues 66-86 of BCS1) fused to the N-terminus of DHFR. Upon incubation with energized mitochondria, a processed form of the preprotein was generated in a $\Delta\Psi$–dependent manner. The processed species was protected from degradation by proteinase K (Fig. 15B). Thus, the chimeric precursor was imported into the mitochondrial matrix where it was processed by MPP. Hence, residues 66-86 of BCS1 behave like a typical mitochondrial targeting sequence (MTS).

Next, the interactions of this presequence-like segment with the TOM complex were studied. Antibodies against Tom components could precipitated the import intermediates upon incubation of BCS1(66-86)-DHFR with OMV (Fig. 15C).

Furthermore, when chemical cross-linking was performed under these conditions specific cross-linking adducts were formed between BCS1(66-86)-DHFR and both Tom70 and Tom40 (Fig. 15D). Thus, the interactions of this segment with the mitochondrial outer membrane are mediated by the TOM complex.

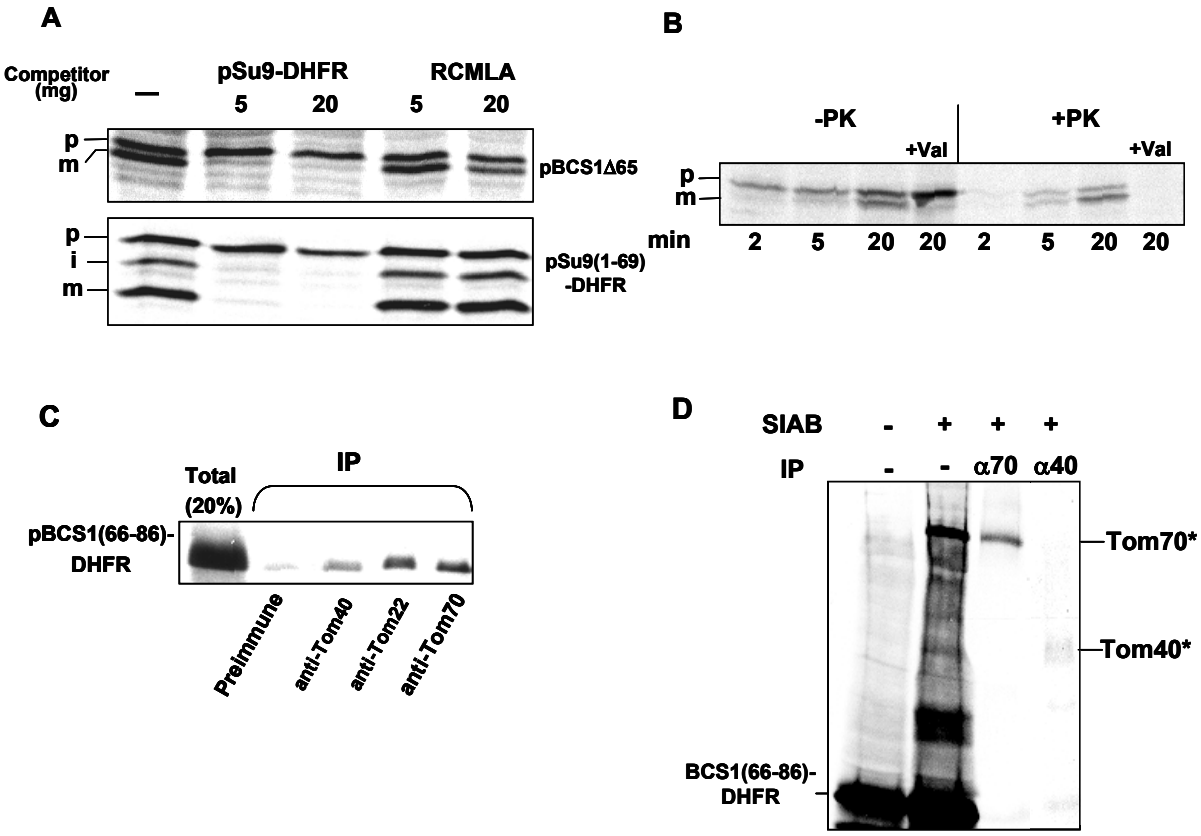

Fig. 15. The sequence comprising residues 66-86 containing a presequence and interacts with Tom components. (A) Radiolabelled pBCS1Δ65 and, as a control, pSu9(1-69)-DHFR were incubated for 20 min at 15°C in SI buffer with either mitochondria ("-") or with mitochondria preincubated with the indicated amounts of proteins for 2 min on ice. At the end of the import reactions mitochondria were washed, reisolated, and analyzed by SDS-PAGE. p, precursor; i, intermediate size form; m, mature form. **(B)** Residues 66-86 can direct a cytosolic protein into the mitochondrial matrix. Radiolabelled BCS1(66-86)-DHFR was incubated at 25°C for the indicated time periods with mitochondria in SI buffer, in the absence or presence of valinomycin (1 μM). Mitochondria were reisolated, resuspended in SEM and divided into two halves. One half was left on ice (-PK) while the other was treated with proteinase K (+PK). The import reactions were analyzed by SDS–PAGE. Precursor and mature forms are indicated by p and m, respectively. **(C)** Residues 66-86 can promote interaction with the TOM complex. Radiolabelled pBCS1(66-86)-DHFR was incubated with OMV for 20 min at 25°C. The OMV were washed with a buffer containing 100 mM KCl, re-isolated and solubilized in 0.75% ß-DDM containing buffer. Further treatment and co-immunoprecipitation were as described in the legend to Fig. 9B. **(D)** Radiolabelled BCS1(66-86)-DHFR precursor was incubated in the presence of MTX/NADPH with isolated OMV for 2 min at 0°C, followed by incubation for 5 min at 25°C. The chemical cross-linker N-succinimidyl (4-iodoacetyl) aminobenzate (SIAB) was then added for further 40 min at 10°C. Cross-linked samples were immunoprecipitated with antibodies against Tom70 or Tom40. Cross-link adducts of BCS1 to Tom proteins are labelled.

Does every MTS allow the BCS1 precursor to reach its functional location? A BCS1 variant where residues 69-83 were replaced by residues 1-48 of Su9, a well-studied

presequence segment, was constructed. Surprisingly, the resulting precursor, Bcs1-Su9 was imported *in vitro* into mitochondria where most of it was processed by MPP (Fig. 16A).

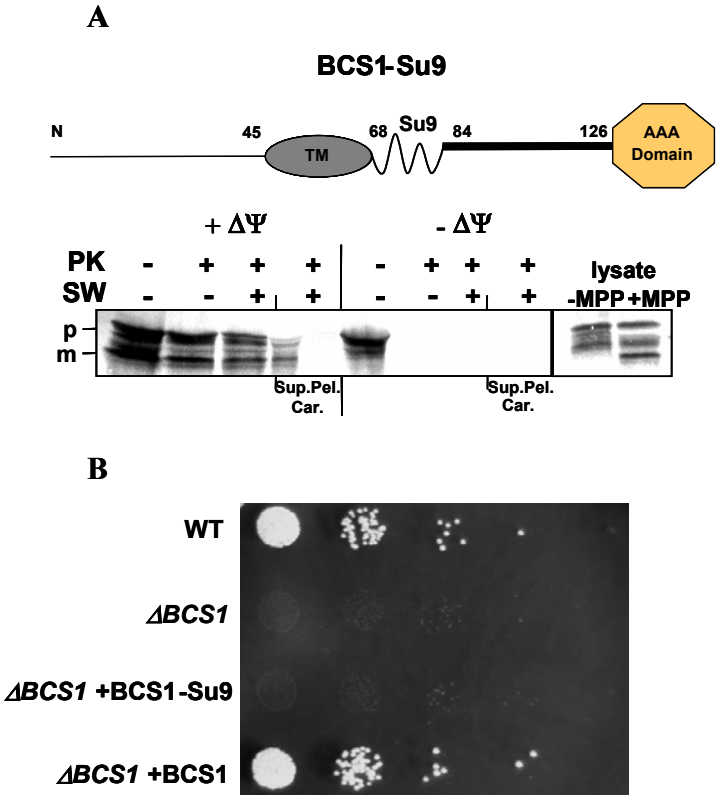

Fig. 16. Residues 66-86 of BCS1 cannot be replaced by another presequence. (A) BCS1-Su9 is not imported *in vitro* properly to the mitochondrial inner membrane. Radiolabelled BCS1-Su9 (see scheme) was incubated with isolated yeast mitochondria in the absence or presence of 1 μM valinomycin for 20 min at 25°C (+ΔΨ or -ΔΨ, respectively). Further treatment was as described in the legend to Fig. 15A. A sample that was swelled and treated with proteinase K after import was subjected after reisolation to carbonate extraction. Supernatant (Sup.) and pellet (Pel.) were loaded on the gel. To recognize the processed form of BCS1-Su9 the radiolabelled precursor was incubated in the presence or absence of recombinant MPP and the proteins were analyzed by SDS-PAGE (right panel) **(B)** BCS1-Su9 cannot functionally replaced BCS1. *BCS1Δ* cells, their isogenic wild type, and *BCS1Δ* cells transformed with either BCS1 coding sequence or sequence encoding BCS1-Su9 were tested by dilutions in 10-fold increments for their ability to grow at 30°C on YPG medium.

The identity of the lower band as the processed species was verified by treating the radiolabelled precursor with recombinant MPP (Fig. 16A, right panel). The import into the matrix was dependent on ΔΨ; in its absence the protein accumulated on the mitochondrial surface (Fig. 16A). However, the typical fragments of native BCS1 were not formed after disruption of the outer membrane and protease treatment. Apparently, instead of being inserted into the inner membrane this construct was mis-sorted to the matrix. Replacing the

presequence-like helix by a shorter presequence, namely amino acid residues 1-22 from CoxIV (Hines *et al.,* 1990) resulted also in mis-sorting of the protein without formation of the typical fragments of native BCS1 (data not shown). These findings were supported by *in vivo* experiments. A *BCS1* null strain was transformed with yeast expression vector containing either Bcs1-Su9 or Bcs1-CoxIV. Both variants could not rescue the phenotype of a *BCS1* null strain and support growth on YPG (Fig. 16B, ΔBCS1+BCS1-Su9). Thus, presequence-like domain of BCS1 cannot be replaced by another matrix targeting signal-type signal.

The role of the auxiliary import sequence of BCS1

An unexpected result of the peptide scan was the strong binding of all three receptors to the region of amino acid residues 91-126. Does this binding reflect physiologically meaningful binding to the TOM complex? A construct lacking both the transmembrane domain and the presequence-like helix, BCS1Δ82, was still able to bind the TOM complex with an efficiency similar to that of the wild type construct (Fig. 17A). This binding was reduced upon tryptic removal of the exposed cytosolic domains of the receptor proteins (Fig. 17A). A cytosolic protein, DHFR displayed only background levels of binding under these conditions (data not shown).

To further study the capacity of residues 84-126 of BCS1 to bind the TOM complex, a fusion protein consisting of this region and of the cytosolic protein DHFR (BCS1(84-126)-DHFR) was incubated with OMV. Specific binding to the TOM complex was observed (Fig. 17B). This binding could be reduced by more than 40% upon competition with chemical amounts of matrix-targeted precursor. The binding of a typical matrix-destined radiolabelled precursor, pSu9-DHFR is presented for comparison (Fig. 17B). The ability of amino acid residues 84-126 to be recognized by the TOM complex was further verified by co-immunoprecipitation. Upon incubation of the fusion protein BCS1(84-126)-DHFR with OMV, antibodies against both Tom20 and Tom40 could precipitate the radiolabelled protein (Fig. 17C).

This suggests a tight interaction of the fusion protein with the TOM complex. When OMV were pretreated with trypsin to remove the cytosolic domains of the import receptors and then incubated with BCS1(84-126)-DHFR, a reduced level of binding was observed. Nevertheless, under these conditions the precursor was also attached to the pore-forming component Tom40 (data not shown). Thus, this domain interacts not only with the receptors but also with the core components of the TOM complex.

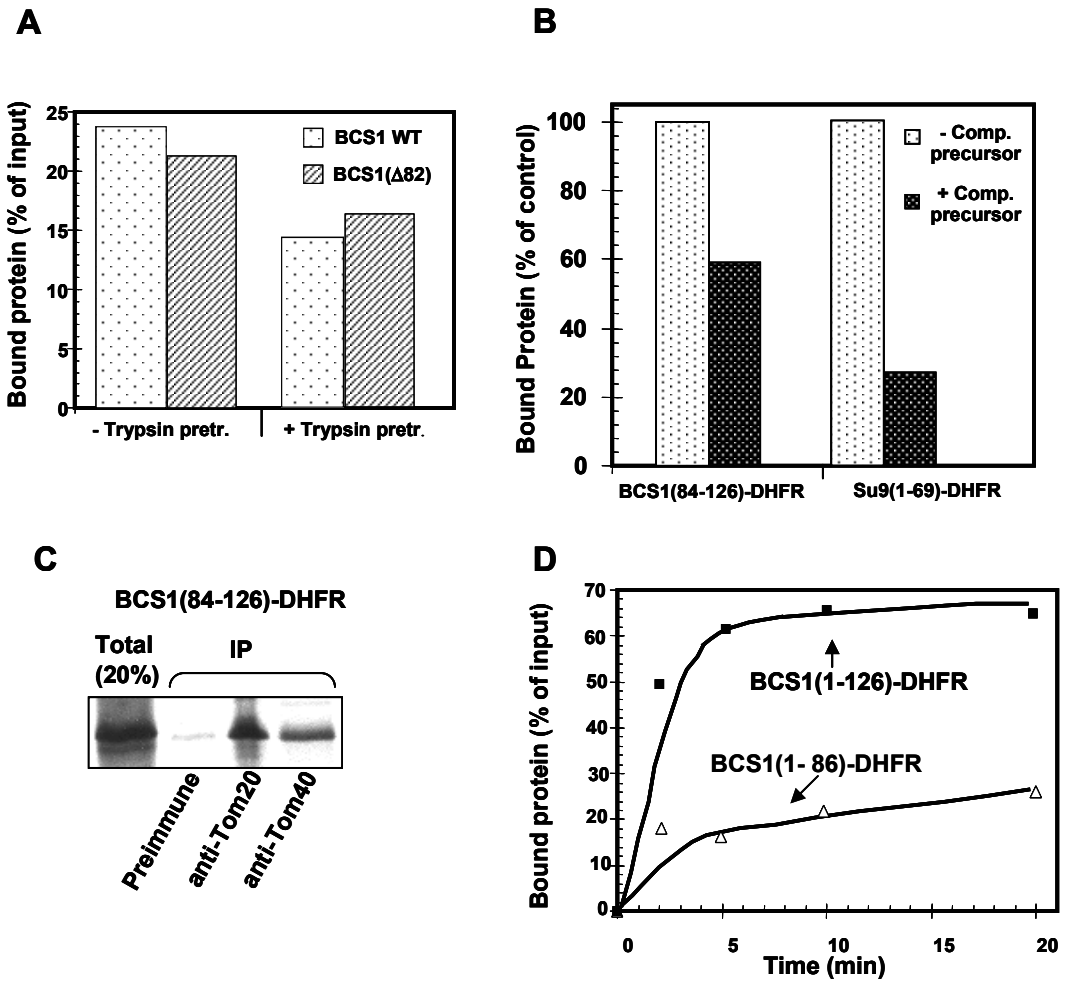

Fig. 17. The sequence comprising residues 84-126 of BCS1 can promote binding to the TOM complex. (A) Precursor lacking the first 82 amino acid residues of BCS1 can bind to OMV in a receptor-dependent manner. Radiolabelled BCS1 and BCS1(Δ82) were incubated for 20 min at 25°C with either intact OMV or OMV pretreated with trypsin. OMV were then washed with buffer containing 200 mM KCl, reisolated, and analyzed by SDS-PAGE and phosphorimaging. The average of three different experiments is presented. **(B)** Residues 84-126 of BCS1 promote binding to the TOM complex. Radiolabelled precursors of BCS1(84-126)-DHFR or pSu9(1-69)-DHFR for comparison were incubated for 20 min at 0°C with OMV in the absence ("-Comp. precursor") or presence of chemical amounts of pSu9(1-69)-DHFR ("+Comp. precursor"). Further treatment was as in (A). The amount of protein bound to untreated OMV was set to 100%. **(C)** BCS1(84-126)-DHFR bound to OMV can be co-immunoprecipitated with components of the TOM complex. Radiolabelled precursor was incubated for 20 min at 25°C with OMV. The OMV were then treated with buffer containing 100 mM KCl. OMV were reisolated, pelleted and resuspended in SEM buffer. Immunoprecipitation (IP) was performed with antibodies raised against Tom20, Tom40 or with preimmune serum. To control for binding, an aliquot was removed before the co-immunoprecipitation and precipitated with TCA (Total). **(D)** Residues 87-126 of BCS1 increase binding to OMV. BCS1(1-86)-DHFR and BCS1(1-126)-DHFR were incubated with OMV in the presence of MTX/NADPH at 15°C for the indicated time periods. OMV were then washed with buffer containing 20 mM KCl, reisolated, dissolved in sample buffer, and analyzed by SDS-PAGE and phosphorimaging. For each protein the amount of radiolabelled precursor added to the reaction was set to 100%.

To test the contribution to binding of residues 87-126 in the context of the full sequence, the binding to OMV of BCS1(1-86)-DHFR was compared to that of BCS1(1-126)-DHFR. The longer construct had a three fold higher binding capacity demonstrating the auxiliary effect of residues 87-126 (Fig. 17D). Hence, the strong binding of Tom components to residues 91-126 of BCS1 reflects a physiological affinity. In agreement with this observation, a construct lacking the first 82 amino acid residues of BCS1 was still able to bind to the mitochondrial outer membrane, and in addition, amino acid residues 84-126 were found to be necessary for sorting to the inner membrane (Fölsch *at al.,* 1996).

Thus, amino acid residues 84-126 play an important role in the import of BCS1; this region is involved in both binding to the TOM complex and in correct sorting to the inner membrane.

3.2.6. BCS1 does not require soluble intermembrane space components for correct import

Most precursors without N-terminal targeting signals, such as the members of the solute carrier family, require the presence of soluble factors, Tim9 and Tim10 in the intermembrane space, for their import into the inner membrane (Koehler *et al.,* 1998; Sirrenberg *et al.,* 1998). These precursors cannot be imported into the inner membrane in mitoplasts, i.e. when the outer membrane of mitochondria is disrupted and these soluble factors are released (Kübrich *et al.,* 1998). Does the precursor of BCS1 also require these or other factors in the intermembrane space? When the BCS1 precursor was imported into mitoplasts insertion of the precursor into the inner membrane was not impaired, as verified by the formation of characteristic proteolytic fragments (Fig. 18). Hence, after eliminating the physical barrier of the outer membrane BCS1 can be imported directly into the inner membrane in a process that probably does not require soluble factors in the intermembrane space.

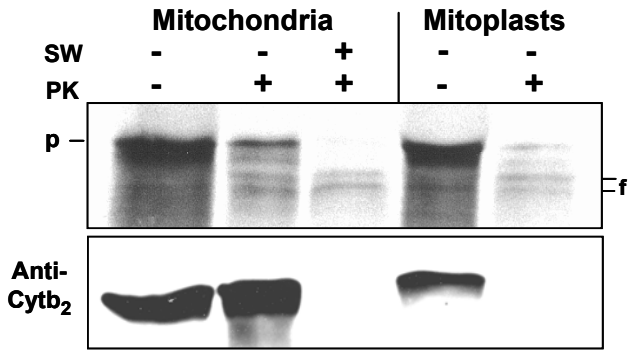

Fig. 18. BCS1 can be imported into mitochondria with ruptured outer membrane. Radiolabelled BCS1 was imported into yeast mitochondria at 25°C. Each sample was split into three aliquots and further treatment was as described in Fig. 14A. For import into ruptured mitochondria (mitoplasts) the organelles were first incubated for 30 min at 0°C in 20 mM Hepes, pH 7.2. Mitoplasts were then reisolated, resuspended in SI buffer, and import was performed. Samples were then split into two halves that were diluted 10 times with either SH buffer, or with SH buffer containing proteinase K (+PK). In the end of all import reactions mitochondria or mitoplasts were reisolated. The pellets were resuspended in sample buffer and import was analyzed by SDS–PAGE. P, precursor protein; f, fragments generated by proteinase K treatment of mitoplasts. Immunobloting of the endogenous intermembrane space protein Cytochrome b_2 was performed to control for mitoplasts formation and is presented in the lower panel.

3.2.7. BCS1 precursor crosses the TOM complex in a loop structure

Proteins of the carrier family and Tim23 pass through the TOM complex in a loop structure (Ryan *et al.,* 1999; Wiedemann *et al.,* 2001; Curran *et al.,* 2002). To find out whether BCS1, as another protein with an internal import signal, crosses the TOM complex in a similar manner, a protein where DHFR moieties are fused at both termini of BCS1 was constructed. The radiolabelled protein DHFR-BCS1(1-250)-DHFR was incubated with energized mitochondria in the presence of MTX which stabilizes the folded conformation of DHFR and prevents its translocation across the outer membrane. When increasing amounts of trypsin were added under these conditions, specific proteolytic fragments in the size of 14-16 kDa were formed (Fig. 19A). No such fragments were generated when BCS1 was treated with trypsin in the absence of mitochondria. In this case only the folded DHFR domain was protected from degradation (Fig. 19A). Thus, the fragments are import-specific. The protected fragments remained attached to mitochondria when the organelles were centrifuged after trypsin treatment. In contrast, the folded DHFR was released to the supernatant (Fig. 19B). Hence, protected fragments reflect a situation where the internal import signal is imported into the mitochondria while both termini are still exposed at the outer membrane.

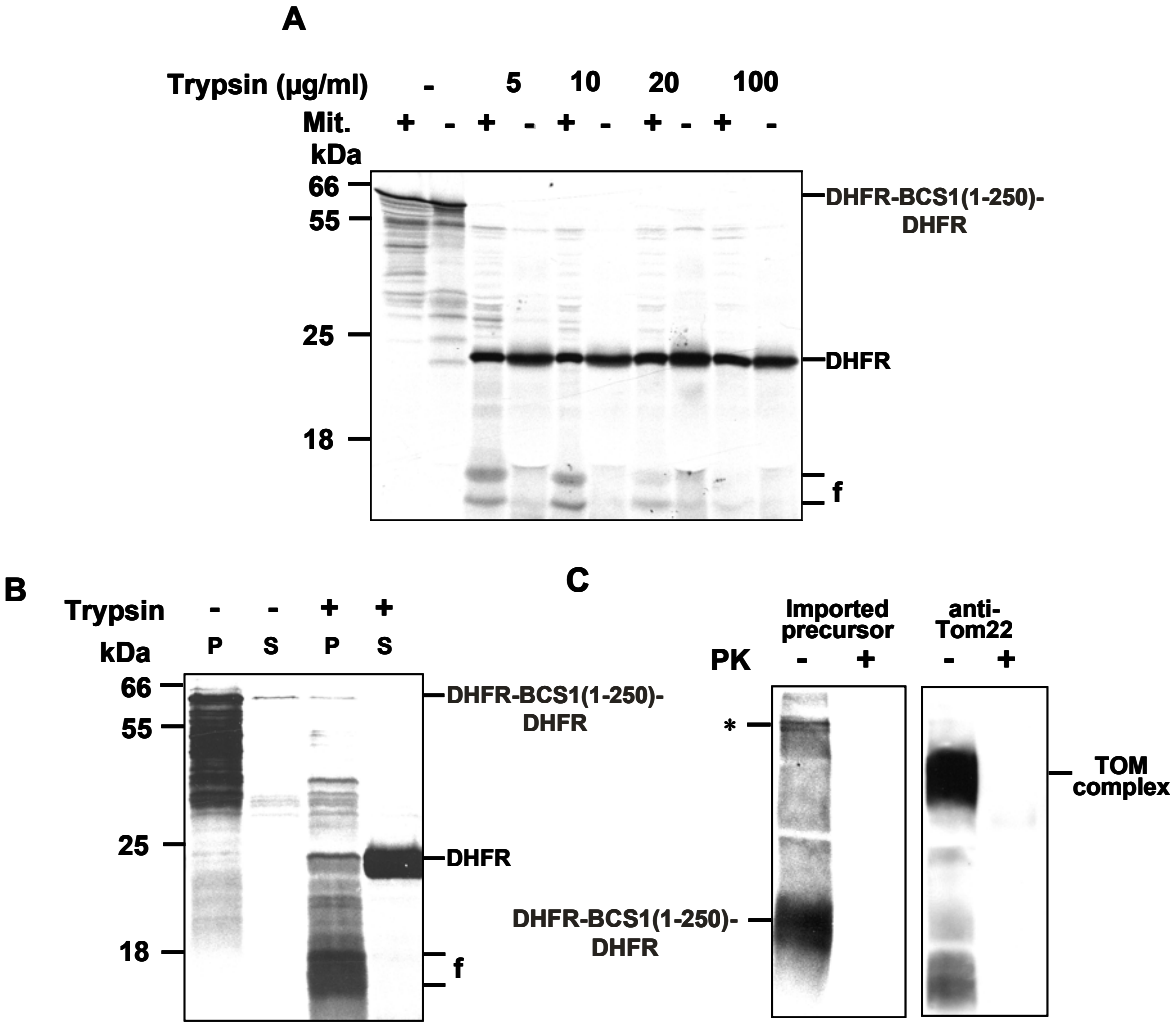

Fig. 19. BCS1 passes the TOM complex as a loop structure. (A) Radiolabelled DHFR-BCS1(1-250)-DHFR was incubated in a MTX/NADPH-containing F5 buffer with mitochondria for 20 min at 25°C. The mitochondria were washed in a buffer containing 20 mM salt, resuspended in a SEM buffer and treated with the indicated concentration of trypsin for 15 min on ice. After inhibition of the protease by PMSF samples were precipitated by TCA and analyzed by SDS-PAGE. The bands corresponding to the DHFR domain and the proteolytic fragments (f) are indicated **(B)** Radiolabelled DHFR-BCS1(1-250)-DHFR was incubated with mitochondria as described above for part (A). After treatment with trypsin (5 µg/ml), the mitochondria were re-isolated. Whereas pellets were dissolved directly in sample buffer, the supernatant was precipitated first with TCA. All samples were analyzed by SDS-PAGE. The bands corresponding to the DHFR domain and the proteolytic fragments (f) are indicated **(C)** Radiolabelled DHFR-BCS1(1-250)-DHFR was incubated in a MTX/NADPH-containing SI buffer with mitochondria for 20 min at 25°C. The samples were halved and while one aliquot was treated with proteinase K (20 µg/ml) the other was left intact. The mitochondria were centrifuged, dissolved in buffer containing 0.4% digitonin and analyzed by BNGE. The left panel shows the autoradiography while the right panel presents immunodecoration of the same membrane with antibody against the cytosolic domain of Tom22. The radiolabelled precursor migrating with the TOM complex is indicated with an asterix.

BNGE was used to study if the import intermediate of DHFR-BCS1(1-250)-DHFR is attached to the TOM complex. Upon import into isolated energized mitochondria a part of the precursor was found to migrate as a high molecular weight complex (Fig. 19C). As expected, the complex containing the accumulated radiolabelled precursor is larger than the TOM complex, since the mass of the precursor is added to that of the endogenous TOM machinery. The observation that only part of the precursor migrated together with the TOM complex is not surprising considering the fact that even known components of the TOM complex, like Tom20 and Tom70, dissociate from the complex while performing BNGE (Dekker *et al.,* 1997). To demonstrate that the BCS1 precursor was accumulated at the outer membrane, the accessibility of the precursor protein to proteinase K was investigated. The imported precursor, like the cytosolic domain of Tom22, was completely digested (Fig. 19C). Hence, DHFR-BCS1(1-250)-DHFR accumulates at the outer membrane, most probably at the TOM complex.

4. DISCUSSION

4.1. *Preprotein interaction with the TOM complex*

To better understand how precursor proteins are recognized by the mitochondria and translocated across the outer membrane, I have analyzed the interaction of mitochondrial precursor proteins with the TOM complex by studying purified components. Precursors were found to bind efficiently to the isolated complex in the absence of chaperones and lipids in a bilayer structure. Presequences were transferred into the translocation pore in such a manner that they became inaccessible to added mitochondrial processing peptidase, MPP. Thus, the TOM complex isolated from the mitochondria represents the minimal machinery for the recognition and partial translocation of precursor proteins with N-terminal extensions. Similar binding characteristics were described for a protein import complex which was obtained after solubilizing chloroplast outer envelopes with mild detergent (Soll and Waegemann, 1992).

Previous reports suggested that purified domains of Tom components are able to bind precursor proteins (Brix *et al*., 1997; Schleiff *et al*., 1997; Komiya *et al*., 1998). In those experiments recombinant soluble domains of Tom receptor proteins were incubated with sub-stochiometric amounts of precursor proteins synthesized in cell free systems which contained chaperones and potential presequence binding factors. Furthermore, the presence of cytosolic chaperones was suggested to be essential for the binding of mitochondrial precursors to the cytosolic domains of Tom70 and of Tom20 (Komiya *et al*., 1997). It was shown that the purified TOM complex reconstituted into artificial vesicles could facilitate membrane insertion of resident outer membrane proteins (Tom40, Tom70 and porin), translocation of cytochrome *c* heme lyase, and translocation of the N-terminal targeting sequence of a protein targeted to the matrix. These experiments were performed with precursor proteins synthesized in cell free systems which contained chaperones and potential presequence binding factors (Künkele *et al.,* 1998). Using fluorescence correlation spectroscopy (FCS), the equilibrium binding constant of fluorescently labelled precursor protein with isolated TOM complex was measured (Stan *et al.,* 2000). The value obtained in the nM range suggests a relatively high affinity, significantly higher than the affinities of the same precursor to soluble domains of Tom20 or Tom70 that were measured with the surface plasmon resonance technique (Iwata and Nakai, 1998). The observed binding with isolated components suggests that chaperones are not essential for targeting or translocation but rather have a stabilizing effect on precursor proteins.

Such binding further demonstrates that in contrast to previous views, interaction

with a bilayer structure is not prerequisite for translocation by the import machinery. Tom components may selectively bind an amphipathic structure of a presequence that is in equilibrium between ordered and disordered states. According to recent structural work, a presequence peptide binds in a hydrophobic groove formed by the cytosolic domain of Tom20, and in this state, is present in an amphipathic helical structure (Abe *et al*., 2000).

In addition to recognition, the purified complex is able to transfer the presequence part from an exposed location at the complex to a location inaccessible to added MPP, likely into the translocation pore. Insertion of the presequence into the import pore can occur in the absence of an external energy source and receptors. Increasing affinity of presequence binding sites may lead to a vectorial movement of the precursor from the surface to the pore (Mayer *et al*., 1995c; Komiya *et al*., 1998; Rapaport *et al*., 1998b). Precursor molecules approaching the mitochondrial outer membrane are normally bound first to low affinity binding sites at the *cis* side of the membrane and are then transferred to sites with higher affinity on the *trans* side of the membrane (Mayer *et al*., 1995b; Mayer *et al*., 1995c; Rapaport *et al*., 1998b). The observed lower affinity of precursors for soluble receptor domains, as compared to the high affinity measured for the TOM complex supports this view (Iwata and Nakai, 1998; Abe *et al*., 2000). Stable interaction of the presequence with the *trans* side and inaccessibility of the MPP cleavage site at the *cis* side of the outer membrane, are accompanied by unfolding of the mature part of the preprotein if this is situated close to the targeting sequence (Mayer *et al*., 1995c; Rapaport *et al*., 1998b). Likewise, when the cleavage site of the precursor in the soluble complex was inaccessible to the added MPP, the mature part was unfolded. The TOM complex has to be in a specific conformation to perform this partial translocation, as the core complex that contains the required components was inactive in the absence of lipids. When the soluble domains of the components of the TOM complex were removed by treatment with protease, binding of the preprotein occurred so that the MPP cleavage site still was protected. The environment of the presequence in the protected location is formed mainly by the membrane-embedded parts of the TOM complex. The bulk of these parts are composed by Tom40. These findings are in agreement with previous studies which identified Tom40 as the major component involved in both formation of the translocation pore and binding of precursor proteins (Vestweber *et al*., 1989; Rapaport *et al*., 1997; Hill *et al*., 1998; Künkele *et al*., 1998b; Rapaport *et al*., 1998a). Reconstituted Tom40 could specifically bind mitochondrial precursor proteins (Hill *et al.,* 1998); also, purified Tom40p preferentially recognizes the targeting sequence of mitochondrial precursor proteins (Gordon *et al.,* 2001).

Upon incubation of excess precursor with the TOM core complex, a major part of it remained unbound suggesting that the TOM complex presents a limited number of binding sites. Saturation of the precursor binding sites occurred when eight or nine molecules were bound to one TOM core complex (Stan *et al.,* 2000). Interestingly, the number of precursor binding sites per TOM complex seems to be similar to the estimated number of 8-9 Tom40 molecules present in each TOM complex (Künkele *et al*., 1998a). Thus, each Tom40 molecule could bind one precursor molecule. Binding to the soluble TOM core complex can occur from either the cytosolic or the intermembrane face. It is unclear how many preprotein molecules can bind simultaneously to the TOM complex embedded in the mitochondrial outer membrane. It may be envisaged that the high number of binding sites in the isolated complex is the result of an ability of the presequence to bind at sites which are not accessible when the membrane-integrated complex is analyzed. Another possibility is that all these binding sites are also available in the *in vivo* situation. However, due to a possibly different topological arrangement of the native complex compared to the soluble complex, the interaction of preprotein in transit with one of these sites in vivo may prevent by steric hindrances the binding of other preproteins. A clear answer can come only from structural information on the TOM complex in the presence of preproteins.

4.2. *Interaction of the BCS1 protein with the TOM complex*

4.2.1. Internal targeting signal segments of BCS1 and their recognition by the TOM complex

BCS1 was used as a model protein to investigate what the signals are in a precursor with internal targeting and sorting information and how these signals are are decoded by the mitochondrial TOM complex. Up to now only few internal import signals have been identified (Fölsch *et al.,* 1996; Arnold *et al.,* 1998; Diekert *et al.,* 1999; Egan *et al.,* 1999; Endres *et al.,* 1999; Dembowski *et al.,* 2001). BCS1 is highly conserved among various organisms (Fig. 20). Amino acid residues 1-126 of BCS1 were shown to contain all the required information for targeting and sorting of the protein (Fölsch *et al.,* 1996). Four sequence elements can be identified in this region: (i) the N-terminal residues 1-44, (ii) a transmembrane domain at amino acids 45-68, (iii) a presequence-like helix (residues 69-83), and (iv) an import-auxiliary region at residues 84-126.

The N-terminal region does not play a role in targeting and sorting of the protein. It can be removed without affecting the import efficiency (Fölsch *et al.,* 1996). Furthermore, it is present only in the yeast BCS1 suggesting that it does not play a crucial role in the import or function of the protein.

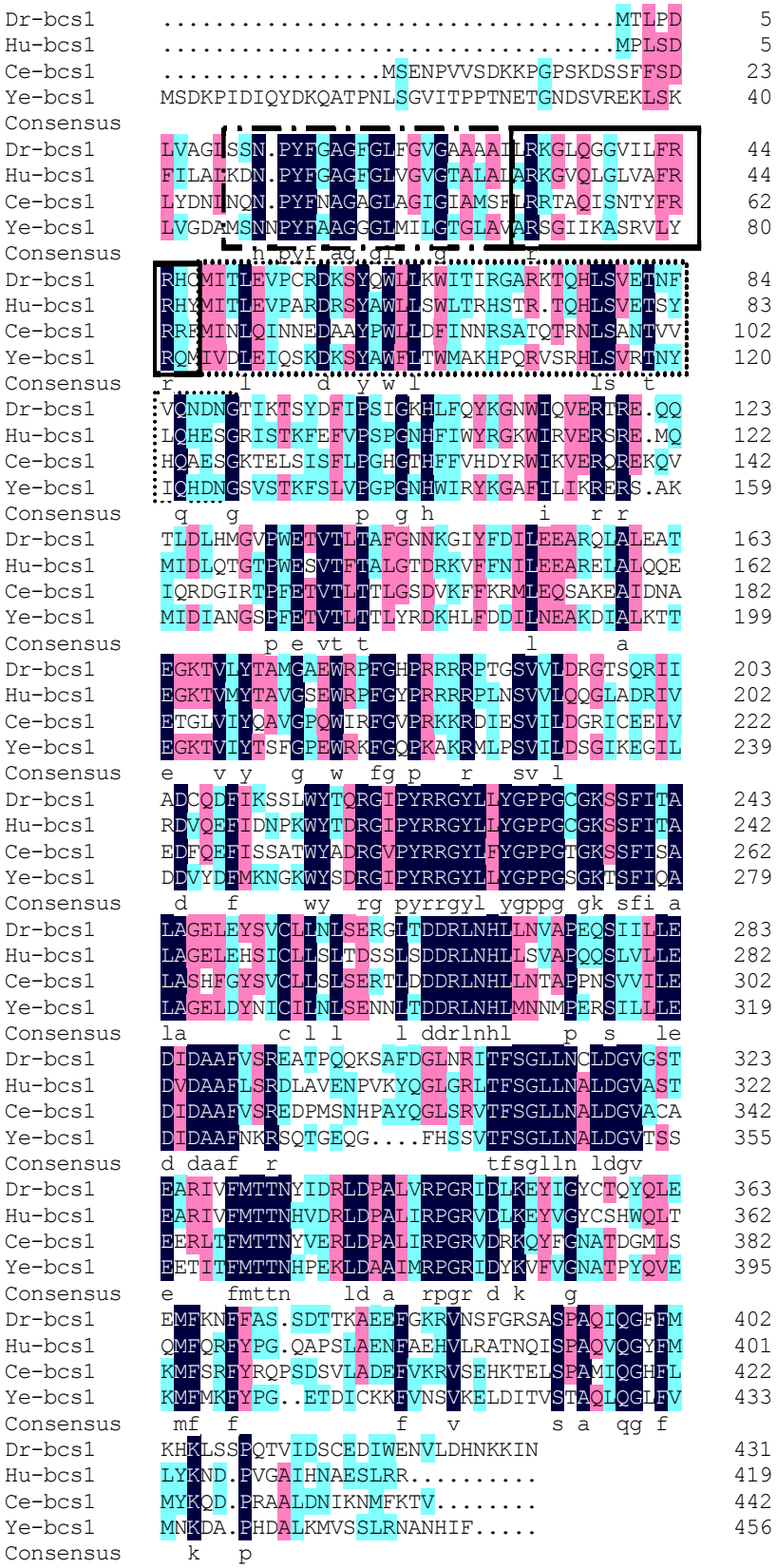

Fig. 20. BCS1 is highly conserved protein. Protein sequence alignment of yeast BCS1 with homologues from other organisms is presented. Identical residues occurring in all four organisms are indicated in black highlight. Similar residues are indicated by gray highlight. Amino acids residues belonging to the transmembrane domain, presequence-like helix and import-auxiliary region are marked with interrupted line, continuous line or dotted line, respectively. Dr, *D. melanogastor*; Hu, *H. sapiens*; Ce, *C. elegans*, Ye; *S. cerevisiae*.

The transmembrane domain is an essential element of the internal import and sorting information of the BCS1 precursor and was found to be highly conserved among various organisms (Fig. 20). Nevertheless, the cytosolic domains of the Tom receptors do not bind to peptides corresponding to this segment. Removing the transmembrane segment or replacing it with a transmembrane segment from another inner membrane protein impaired sorting to the inner membrane but not targeting to the outer membrane and association with the TOM complex (Fölsch *et al.,* 1996 and this thesis). Apparently, the transmembrane domain of BCS1 does not have a role in targeting the precursor to the mitochondrial surface or in interaction with the TOM complex. Rather, it is involved in the sorting and insertion into the mitochondrial inner membrane.

The various components in the mitochondrial import machinery were reported to have different affinities towards transmembrane segments. The cytosolic domains of Tom20 and Tom22 bind very weakly or not at all to peptides representing the transmembrane segments from another inner membrane protein with internal import information, the phosphate carrier (Brix *et al.,* 1997). Tom70 did not bind the transmembrane domain of BCS1 while it binds part of the charged and part of the uncharged peptides of the phosphate carrier. Hence, a hydrophobic character of a segment is not sufficient to ensure its recognition by Tom70. The small Tim components (Tim9-Tim10 complex) display a completely different behavior; they bind the transmembrane segments of the ADP/ATP carrier but also the loops between them (Curran *et al.,* 2002; A. Vasiljev, personal communication). Future studies on the affinity of import components towards other internal import signals will help to draw a more detailed picture of the recognition of hydrophobic stretches within mitochondrial precursors.

The presequence-like helix flanking the C-terminus of the transmembrane domain was bound by the three Tom receptors. Despite its similarity to matrix targeting signals, a replacement of this segment by authentic presequences did not result in correct sorting of the mutated precursor both *in vivo* and *in vitro*. The presequences used for replacement had different lengths than the authentic presequence; we cannot rule out the possibility that the length influenced the functionality of the presequence in the context of the entire targeting information. It seems that the presequence-like helix is probably involved in specific intra- or intermolecular interactions.

Interestingly, the highest binding was observed to peptides corresponding to residues 92-109. The potential of this segment, similar to mitochondrial presequences, to form an α-helix with positive charges on one face may explain the strong binding of import receptors to this conserved segment. While this region was suggested before to have

only a marginal effect on import (Fölsch *et al.,* 1996), the results of this study suggest that, in fact, this region of BCS1 precursor plays an essential role in the translocation of BCS1 across the outer membrane. Precursor lacking this region was not properly inserted into the inner membrane (Fölsch *et al.,* 1996). Moreover, this region can specifically direct the BCS1 precursor from the cytosol to the mitochondrial outer membrane. The importance of this element is further demonstrated by its evolutionary conservation from yeast to Drosophila and human (Fig. 20).

In conclusion, the import and intramitochondrial sorting of BCS1 requires three distinct regions of the protein, the transmembrane segment, the presequence-like helix and an import-auxiliary region. The latter two elements are able to interact simultaneously or sequentially with several Tom components, whereas the initial recognition does not require the transmembrane domain. The auxiliary region represents a novel type of signal with targeting and sorting function. It is decoded by all three known mitochondrial import receptors. Such multiple interactions could increase the efficiency of targeting and mediate "quality control".

4.2.2. The unique recognition and import pathway of the BCS1 protein

The interaction of the TOM complex with BCS1 translocation intermediates is different from its interactions with precursors carrying mitochondrial presequences. A recent study demonstrates that Tom20 binds the hydrophobic side of the amphiphilic helix of the presequence (Abe *et al.,* 2000). Nevertheless, under conditions where binding to surface receptors is prevalent, presequence-containing precursors interact with the TOM complex in a mainly electrostatic manner. BCS1 precursor was observed to interact with the TOM complex with a more hydrophobic character. Despite these different modes of binding, a recombinant preprotein can compete out the import of BCS1. Thus, BCS1 uses the same import pore for passage across the outer membrane as preproteins destined for the matrix.

Like most of the mitochondrial precursors, BCS1 probably interacts with cytosolic chaperones which maintain the preproteins in a translocation-competent conformation (Fig. 21A). The results of this study suggest that BCS1 precursor interacts with the TOM complex initially via residues 69-126 which include the presequence-like segment and the auxiliary region (Fig. 21B). Then parts of the precursor move as a loop structure into and through the TOM complex (Fig. 21C). The pore of the TOM complex is estimated to have a diameter of ca. 25 Å (Hill *et al.,* 1998; Künkele *et al.,* 1998a; Schwartz *et al.,* 1999), and hence could accommodate such a loop structure (Fig. 21C).

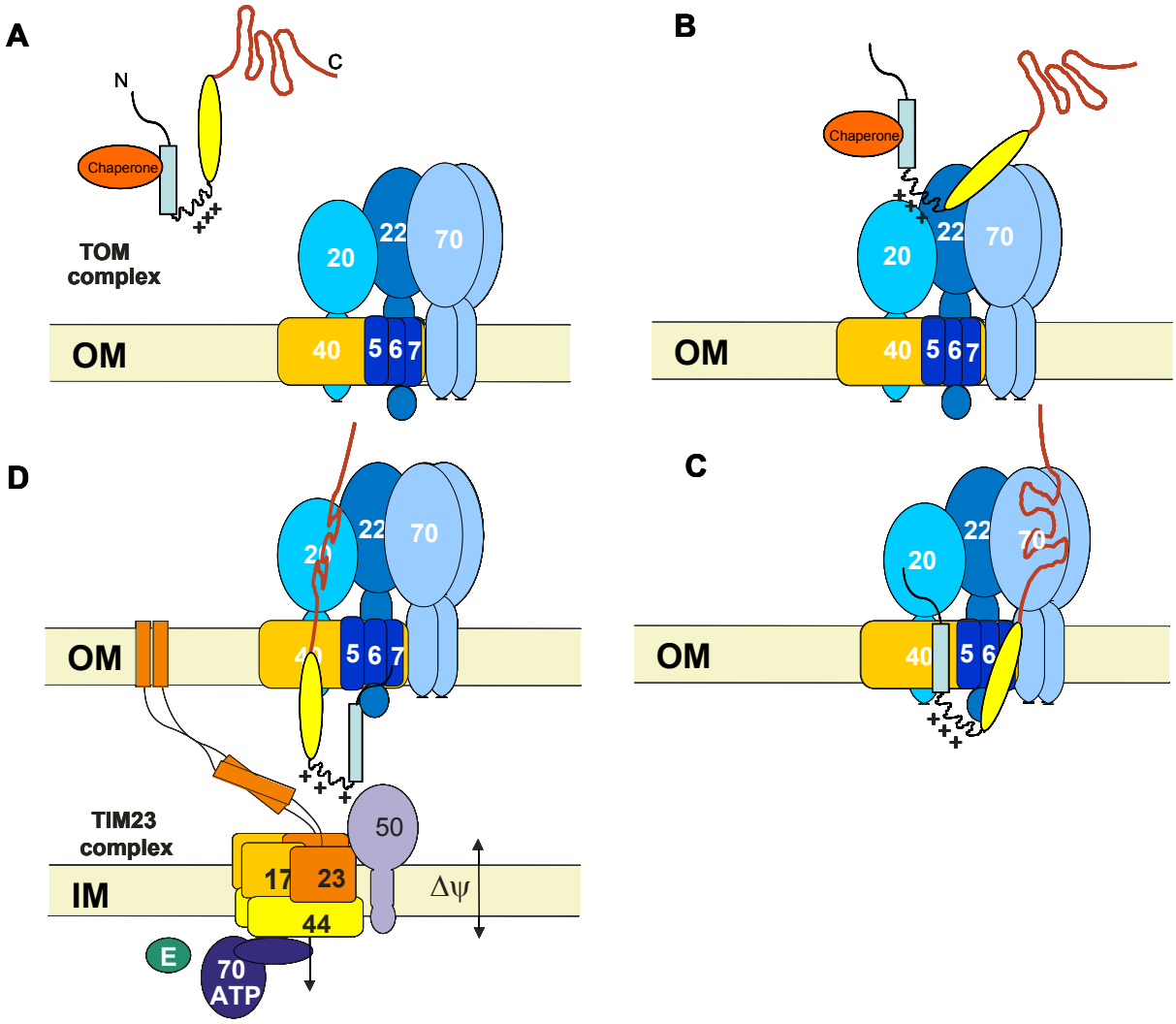

Fig. 21. Model of interaction of BCS1p with the TOM complex. (A) BCS1 precursor is synthesized in the cytosol and kept in a translocation competent conformation through interaction with cytosolic chaperones. Transmembrane domain: cylinder; presequence-like helix: zigzag line; import-auxiliary region: elliptic. **(B)** On its import pathway, BCS1 interacts with all three Tom receptors (Tom20, Tom22 and Tom70). **(C)** BCS1 precursor crosses the TOM complex in a loop structure. **(D)** As the BCS1 precursor emerges from the translocation pore at the TOM complex it is taken over by the TIM23 complex.

Moreover, the precursors of both carrier family proteins and Tim23 were also suggested to be inserted in a loop-wise fashion into the TOM complex (Enders *et al.,* 1999; Wiedemann *et al.,* 2001; Curran *et al.,* 2002). This is in contrast to preproteins with cleavable presequences which enter the TOM complex in a linear fashion with the N-terminal first. Thus, one may speculate that crossing of the TOM complex in a loop structure is a general characteristic of inner membrane proteins with an internal import signal. A possible role of the import-auxiliary region could be to shield the transmembrane domain in order to prevent it from unproductive interactions with parts of the translocation pore. As the BCS1 precursor emerges from the translocation pore at the TOM complex it is

taken over by the TIM23 complex (Fölsch *et al.,* 1996) and all three structural elements (the transmembrane segment, the presequence-like helix and the import-auxiliary region) are essential for the intramitochondrial sorting to the inner membrane (Fig. 21D). Similar to matrix destined precursors, and in contrast to precursors from the carrier family, soluble components in the intermembrane space do not seem to be required for the transfer of BCS1 precursor from the TOM to the TIM23 complex. As was observed for matrix targeted precursors, this process probably involves a combined supra-complex of TOM complex, precursor, and TIM23 complex (Horst *et al.,* 1995; Dekker *et al.,* 1997).

Two models have been proposed to explain BCS1 insertion into the inner membrane (Fölsch *et al.,* 1996). The first one proposes that upon insertion of the hairpin loop into the TIM23 machinery, further translocation could involve passage of only the C-terminal domain into the matrix. The hydrophobic transmembrane domain could partition into the lipid bilayer. Consequently, the N-terminal domain will remain in the intermembrane space. Alternatively, the hairpin loop could insert into the inner membrane and further import would involve the complete translocation of both N- and C- termini across the inner membrane. The N-terminal would undergo a transfer from the matrix side, resulting in the export of the N-terminal tail into the intermembrane space. Experimental data support the last model (Fölsch *et al.,* 1996). After sorting into the inner membrane BCS1 would function as a chaperone in cytochrome bc_1 assembly. It is suggested that BCS1 interacts in an ATP-dependent manner with the cytochrome bc_1 precomplex maintaining it in a state competent for the subsequent assembly of the Rieske FeS protein (Cruciat *et al.,* 1999).

5. SUMMARY

The TOM complex, a multisubunit assembly in the mitochondrial outer membrane, mediates targeting and membrane translocation of virtually all nuclear-encoded mitochondrial preproteins analyzed so far. In the present study the mechanisms by which the TOM complex recognizes different precursor proteins and translocates them across the outer membrane were investigated.

In a first part of study the isolated TOM complex was analyzed for its ability to interact with preproteins with N-terminal targeting signals. The TOM translocase was found to bind precursor proteins efficiently in a specific manner in the absence of chaperones and lipids in a bilayer structure. Following the initial binding, the presequence was transferred into the translocation pore in a step that required unfolding of the mature part of the preprotein. This translocation step was mediated also by protease-treated TOM holo complex that contains almost exclusively Tom40. The TOM core complex consisting of Tom40, Tom22, Tom5, Tom6 and Tom7 represents a molecular machine that can recognize and partially translocate mitochondrial precursor proteins.

In a second part of study the interaction of BCS1 precursor with the TOM complex was investigated. BCS1 belongs to the group of proteins with internal, non-cleavable import signals. The information for import and intramitochondrial sorting of BCS1 was localized to the region consisting of amino acid residues 1-126. Three sequence elements were identified in this region: (i) a transmembrane domain (amino acid residues 45-68), (ii) a presequence-like helix (residues 69-83), and (iii) an import-auxiliary sequence (residues 84-126). The contribution of each of these elements to import was studied. The transmembrane domain was found not to be required for stable binding to the TOM complex. The Tom receptors (Tom70, Tom22 and Tom20), as determined by peptide scan analysis, had no affinity for peptides corresponding to the transmembrane domain. They did interact with the presequence-like helix, yet the highest binding was to the region covering residues 92-126. This latter region represents a novel type of signal with targeting and sorting function. It is recognized by all three known mitochondrial import receptors demonstrating their capacity to decode various targeting signals. The results of the present study suggest that the BCS1 precursor crosses the TOM complex as a loop structure. This is in contrast to preproteins with cleavable presequences which enter the TOM complex in a linear fashion with the N-terminal first. Once the precursor emerges from the TOM complex, all three structural elements are essential for the intramitochondrial sorting to the inner membrane.

6. ABBREVIATIONS:

AAC	ADP/ATP Carrier
Ac	acetate
ADH	alcohol dehydrogenase
Amp	ampicillin
APS	ammonium peroxodisulfate
APO	apoferritin
ATPase	adenosine triphosphatase
β-ME	β-mercaptoethanol
BCS1	cytochrome bc_1 precomplex synthesis mediating protein 1
BSA	bovine serum albumin
Ci	Curie
CCHL	cytochrome *c* heme lyase
DDM	n-dodecyl-β-maltopyranosid
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxid
dNTP	deoxynucleotide triphosphate
DNA	desoxyribonucleic acid
DSS	disuccinimidyl suberate
DTT	dithiotreitol
$\Delta\Psi$	electrical membrane potential
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
ER	endoplasmatic reticulum
F1β	β-subunit of mitochondrial F1-ATPase
FCS	fluorescence correlation spectroscopy
FPLC	fast protein liquid chromatography
GIP	general import pore
HEPES	N-2 hydroxyl piperazine-N`-2-ethane sulphonic acid
hsp	heat shock protein
IgG	immunglobulin G
IM	inner membrane
IMS	intermembrane space
IP	immunoprecipitation
IPTG	isopropyl-β,D-thiogalactopyranoside
KAN	kanamycin
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
LB	Luria broth
mM M7G(5`)ppp(5`)G	7-Methylguanosine triphospate
MOPS	N-morpholinopropane sulphonic acid
MPP	mitochondrial processing peptidase
MSF	mitochondrial import stimulating factor
MTS	matrix targeting signal
MTX	methotrexate
MW	molecular weight
N. crassa	*Neurospora crassa*
NADH	nicotine amide adenine dinucleotide
NADPH	nicotine amide adenine dinucleotide phosphate
Ni-NTA	nickel-nitrilo triacetic acid

NMR	nuclear magnetic resonance
ODx	optical density at x nm
OG	n-octyl-β-D-glucopyranoside
OM	outer membrane
OMV	outer membrane vesicles
PAGE	polyacrylamide gel electrophoresis
PAS	protein A Sepharose
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	preimmune serum
PK	Proteinase K
PMSF	phenylmethylsulfonyfluoride
PVDF	polyvinylidene difluoride
RNase	ribonuclease
RNasin	ribonuclease inhibitor
RCMLA	reduced carboxymethylated lactalbumin
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
STI	soybean trypsin inhibitor
TBS	buffer saline
TCA	trichloroacetic acid
TEMED	N,N,N‘,N‘-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
TOM	translocase of the outer mitochondrial membrane
Tris	tris-(hydroxymethyl)-aminomethane
TX-100	Triton X-100
Vol.	volumes
v/v	volume per volume
w/v	weight per weight
WT	wild type

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