Structure and function of the mitochondrial TIM23 preprotein translocase

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1. INTRODUCTION

1.1. Sorting of proteins within a eukaryotic cell

A eukaryotic cell is structurally and functionally subdivided into membrane-bounded compartments - cell organelles. Each organelle is specialized for a specific set of biochemical reactions and, therefore, contains a different set of proteins. With the exception of few proteins synthesized in mitochondria and chloroplasts, all proteins are synthesized on cytosolic ribosomes and have to be transported to the place where they fulfill their biochemical function. The correct sorting of proteins is essential for the life of any cell and the question how this process is managed by the cell one of the major questions in cell biology. The basic concept of intracellular protein sorting is that proteins have targeting signals, either in cleavable extensions or within their mature parts, and that these signals are different for each organelle (Blobel, 2000). Targeting signals are recognized by specific protein complexes which mediate transport of proteins to their final destination. Each organelle has developed its own specific apparatus for recognition and translocation of preproteins. Research in the last years has revealed that translocases are dynamic, multisubunit complexes of great versatility which do not always conform to the same rules.

1.2. Protein translocation into mitochondria – an overview

Mitochondria contain about 1000 different proteins. Of these, only 8 in yeast and 13 in humans are encoded by the mitochondrial genome. All others are encoded in the nucleus, synthesized in the cytosol and have to be translocated into mitochondria. Protein translocation into mitochondria is complicated by the fact that mitochondria contain four subcompartments: two membranes, the outer and the inner membrane, and two aqueous compartments, the intermembrane space (IMS) and the matrix. Mitochondrial membrane proteins not only have to reach mitochondria but have to be integrated into the right mitochondrial membrane. Soluble matrix proteins have to cross two membranes and the aqueous intermembrane space before they reach their final destination. Even though a lot of information has been obtained about protein translocation into mitochondria in the last years, molecular mechanisms and components which allow such versatility and accuracy are still being discovered (Figure 1).

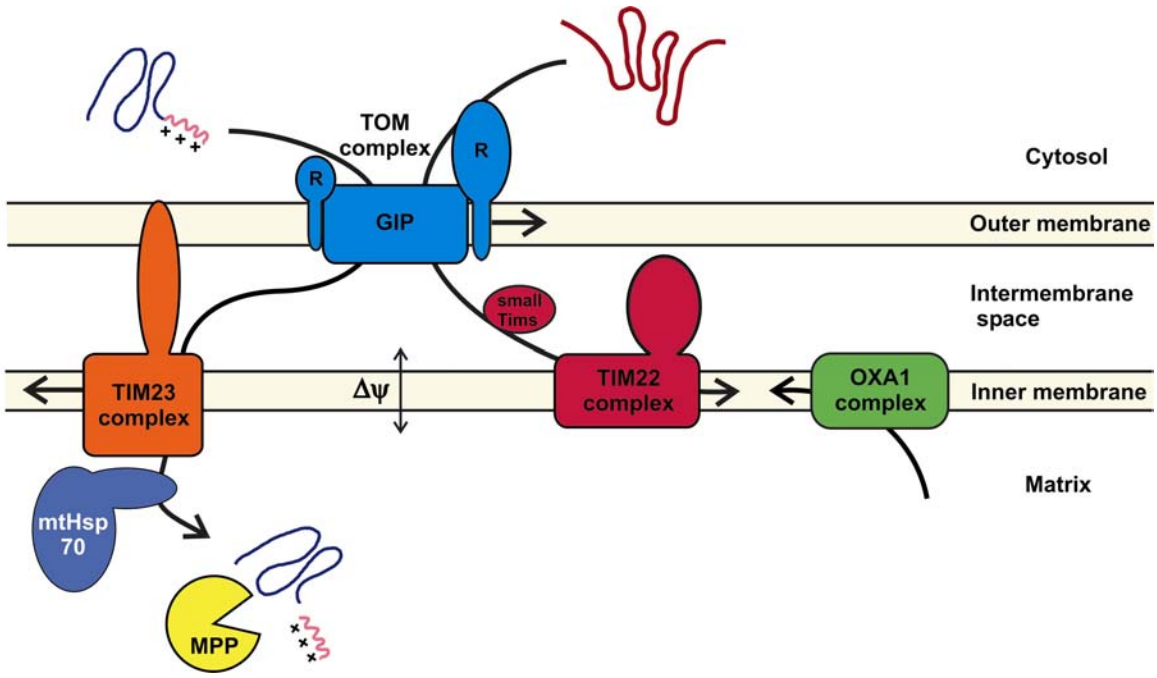

Figure 1. Protein import into mitochondria – an overview

Preproteins carrying an amino-terminal presequence (violet) as well as preproteins with internal targeting signals (red) cross the outer membrane via the TOM complex. Preproteins are first recognized by the receptors (R) which deliver them to the general import pore (GIP). For further translocation of the presequence-containing preproteins, the TOM complex cooperates with the TIM23 complex in the inner membrane. This requires the membrane potential (ΔΨ) and the ATP-dependent action of the mtHsp70. In the matrix, presequences are cleaved off by the mitochondrial processing peptidase (MPP). Preproteins with internal targeting signals are guided by the "small" Tims across the intermembrane space to the TIM22 complex which inserts them into the inner membrane in the ΔΨ-dependent reaction. The Oxa1 complex inserts proteins into the inner membrane from the matrix side.

Proteins are preferentially translocated into mitochondria in a posttranslational manner (Neupert, 1997). Some recent data support the notion that co-translational import contributes to the mitochondrial biogenesis as well (Marc *et al.*, 2002). Newly synthesized mitochondrial precursors are kept in an import-competent conformation in the cytosol by molecular chaperones of the Hsp70 and Hsp90 families, as well as by some specific factors like mitochondrial import stimulation factor (MSF) (Deshaies *et al.*, 1988; Hachiya *et al.*, 1994; Murakami *et al.*, 1988; Young *et al.*, 2003). The most common mitochondrial targeting signal is an N-terminal presequence rich in positively charged amino acid residues which serves as a matrix-targeting signal (MTS). However, a lot of mitochondrial proteins, especially

hydrophobic proteins of the outer and inner membranes, have their targeting signals within the mature part of the polypeptide. Whichever type of signal, all mitochondrial proteins are recognized by the receptors at the mitochondrial surface and are delivered to the translocation channel of the TOM complex (translocase of the outer membrane). Mitochondrial translocases do not allow passage of folded proteins, so precursor proteins have to be in an almost completely extended form to fit into the translocation channels.

After crossing the outer membrane, precursor proteins are directed to one of the translocases of the inner membrane or TIM complexes. All presequence-containing precursors are directed to the TIM23 complex which forms a translocation channel in the inner membrane. The function of the TIM23 translocase requires the membrane potential across the inner membrane. Additional energy input in the form of ATP is needed to complete the translocation process into the matrix. ATP is used by mtHsp70, the central component of the import motor associated with the translocation channel. In the matrix, presequences are cleaved by the mitochondrial processing peptidase (MPP) and the proteins fold into their native form. This process can be supported by the chaperones mtHsp70, Hsp60 and peptidyl-proline *cis/trans* isomerase. Hydrophobic inner membrane proteins follow a different pathway after crossing the outer membrane. With the help of a soluble complex of "small" Tims in the intermembrane space, they are directed to the TIM22 translocase which inserts them into the inner membrane in a membrane potential-dependent manner. Hydrophobic inner membrane proteins encoded in the mitochondrial genome, but also some nuclear encoded ones, are inserted into the inner membrane from the matrix side with the help of the Oxa1 translocase.

1.3. Mitochondrial targeting signals

The most common mitochondrial targeting signal is an N-terminal cleavable presequence which directs a protein into the matrix. When attached to a non-mitochondrial passenger protein, the presequence is necessary and sufficient to target this protein into the mitochondrial matrix. Mitochondrial presequences are usually about 20-60 amino acids long and are rich in positively charged and hydroxylated residues. There seems to be little, if any, sequence conservation among different presequences. What is conserved is their ability to form an amphipathic α-helix with one positively charged and one hydrophobic surface (Roise *et al*., 1988; Von Heijne, 1986). This amphipathic nature is thought to be important for the specific recognition by the mitochondrial import receptors. Indeed, the first high resolution structure of a mitochondrial import component, the receptor Tom20, revealed that the

presequence peptide of the aldehyde dehydrogenase forms an amphipathic helix when bound to Tom20. Tom20 binds to the hydrophobic surface of the helix (Abe *et al*., 2000). The positively charged surface of the helix is probably recognized by the receptor Tom22 (Brix *et al*., 1997).

While the vast majority of the matrix proteins are imported via N-terminal cleavable presequences, few exceptions do exist. Some presequences, for example those of chaperonin 10 and rhodanese, remain attached in the mature protein (Miller *et al*., 1991; Rospert *et al*., 1993). Even more peculiar is the import signal of the DNA helicase Hmi1. It is the only so far reported example of a matrix protein imported via a C-terminal cleavable presequence (Lee *et al*., 1999). Furthermore, it appears that this protein is imported in a C- to N-terminal direction.

Positively charged presequences are also used by some membrane proteins. In these cases presequence-like signals are combined with more hydrophobic segments which lead to an arrest at the level of the outer or inner membrane. These stop-transfer signals are specifically recognized by components of the TOM and TIM23 complexes but how and by which ones is still unknown. Outer membrane proteins, such as Tom20, Tom70 and OM45, have a hydrophilic, positively charged segment followed by the transmembrane domain which together serve as the mitochondrial targeting signal (Rapaport, 2003). Some inner membrane proteins are directed to the matrix via a classical presequence but the mature part of the protein contains a hydrophobic stretch which arrests translocation at the level of the TIM23 channel and the protein is laterally sorted into the inner membrane (Gartner *et al*., 1995; Rojo *et al*., 1998). A presequence-like signal is also found directly at the C terminus of the hydrophobic segment of some inner membrane proteins (Foelsch *et al*., 1996; Westermann and Neupert, 1997). In these cases, the hydrophobic stretch and the presequence-like signal were proposed to form a hairpin-loop structure during import (Foelsch *et al*., 1996). Some proteins of the inner membrane are first completely imported into the matrix via typical presequences and then redirected to the inner membrane (Hartl *et al*., 1986; Herrmann *et al*., 1997).

Members of the carrier protein family of the inner membrane contain targeting signals within the mature protein. These internal targeting signals are not well characterized and seem to be spread throughout the protein (Brix *et al*., 1997; Curran *et al*., 2002a; Endres *et al*., 1999). Even less is known about the targeting signals in the intermembrane space proteins. Some of them use bipartite signals (Glick *et al*., 1992; Hahne *et al*., 1994). In these signals, a typical presequence is followed by a hydrophobic stretch which mediates lateral insertion into the inner membrane and the signal is removed at the outer surface of the inner membrane by a

heterodimeric inner membrane peptidase (Imp1-Imp2) thus releasing the mature protein into the IMS. However, the majority of IMS proteins are imported directly into the IMS through the TOM complex without any involvement of the TIM machineries. Even though the import signals are not clear, mutations which prevent subsequent folding in the IMS diminish the translocation ability of these precursors (Lutz *et al*., 2003).

1.4. Translocase of the outer membrane

The translocase of the outer membrane recognizes virtually all nuclear-encoded mitochondrial preproteins and mediates their translocation across or insertion into the outer membrane (Figure 2). Purified TOM complex consists of seven subunits: three receptors (Tom20, Tom22 and Tom70), a channel forming subunit (Tom40) and three small Tom proteins (Tom5, Tom6 and Tom7) (Kunkele *et al*., 1998). The receptors Tom20 and Tom70 are not stably associated with the core complex which is formed by the rest of the subunits (Ahting *et al.*, 1999; Model *et al*., 2002).

The TOM complex contains multiple binding sites for mitochondrial preproteins on both the cytosolic and the IMS sides of the outer membrane. The cytosolic or the *cis* binding site is mainly formed by receptors Tom20, Tom22 and Tom70. Tom20 is the main receptor for presequence-containing preproteins while Tom70 mainly recognizes internal targeting signals in the precursors of the carrier family (Brix *et al*., 2000; Hines *et al*., 1990; Hines and Schatz, 1993; Moczko *et al*., 1994; Sollner *et al*., 1989; Sollner *et al*., 1990). The observation that the deletion of either of the two receptors is tolerated but the double deletion is not suggests that they are having partially overlapping functions (Lithgow *et al*., 1994; Ramage *et al*., 1993). After initial recognition by the receptors, preproteins are delivered to Tom22 which serves both as a receptor and as a docking point for the other two receptors at the translocation channel (van Wilpe *et al*., 1999). After crossing the translocation channel, preproteins are bound to the *trans* binding site of the TOM complex which is probably formed by the IMS domain of Tom22 together with the IMS exposed loops of Tom40 (Court *et al*., 1996; Moczko *et al*., 1997; Rapaport *et al*., 1997).

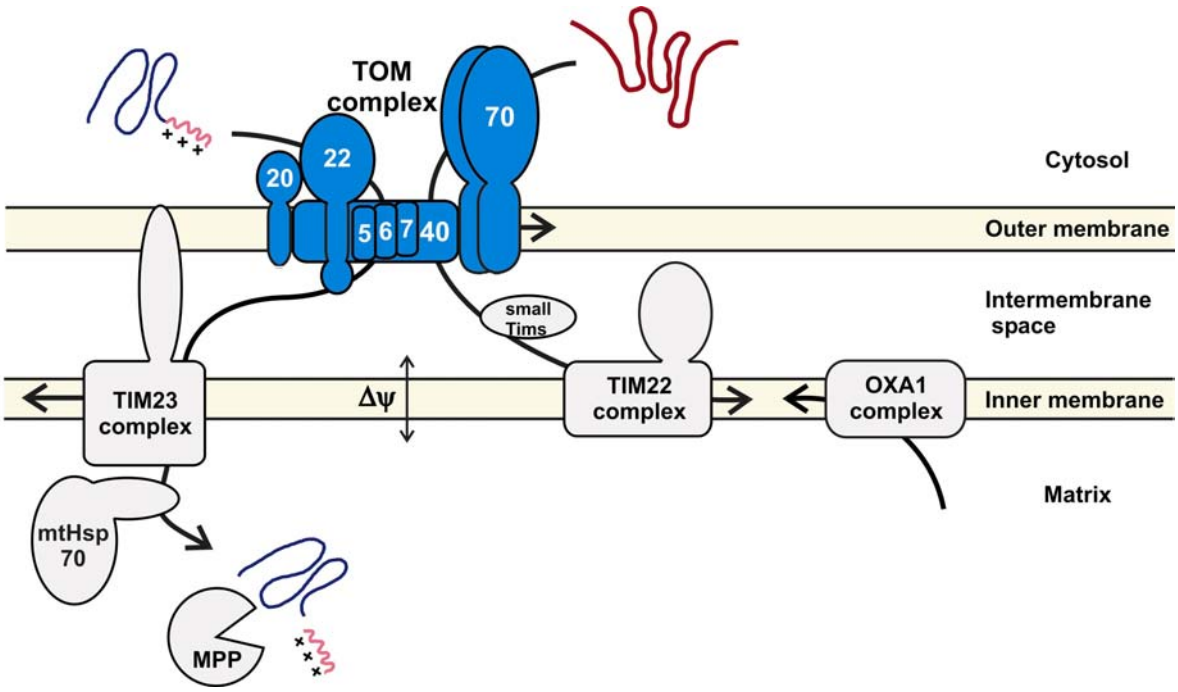

Figure 2. The translocase of the outer membrane (TOM complex)

The TOM complex is responsible for the translocation of virtually all mitochondrial proteins across the outer membrane. It contains two receptors, Tom20 and Tom70, which are loosely associated with the TOM core complex. The TOM core complex contains the third receptor, Tom22, the channel forming subunit, Tom40, and three small proteins of not well understood functions, Tom5, Tom6 and Tom7.

Tom40 is the only essential component of the TOM complex and the only component needed to form the translocation channel of the outer membrane (Ahting *et al*., 2001; Hill *et al.*, 1998). Purified Tom40, either isolated native from mitochondria or renatured after expression in bacteria, forms a voltage-gated, cation-selective high conductance channel which binds mitochondrial presequences. The Tom40 channel appears not to be a passive channel but rather have an active role during translocation (Esaki *et al*., 2003; Gabriel *et al*., 2003).

The function of the small Tom proteins is only partially understood. Tom5 was proposed to act as a receptor following initial receptors Tom20 and Tom22 and also required for the transfer of preproteins from Tom22 to the translocation channel (Dietmeier *et al*., 1997). However some recent data speak against this initial hypothesis (Horie *et al*., 2003). Tom6 and Tom7 appear to be involved in the dynamics of the TOM complex in a partially antagonistic manner (Model *et al*., 2001; van Wilpe *et al*., 1999).

Electron microscopy and 3D reconstruction analysis of the purified TOM complex from *Neurospora crassa* and recently from *Saccharomyces cerevisiae* revealed that it contains two to three pores (Ahting *et al*., 1999; Kunkele *et al*., 1998; Model *et al*., 2002). Each pore probably represents a protein-conducting channel. The pore diameter of ca. 2.5 nm is large enough to accommodate an unfolded or largely unfolded polypeptide chain. Interestingly, imaging of purified Tom40 alone revealed mostly single pore particles confirming that the other subunits are needed for the formation of a higher oligomeric complex (Ahting *et al*., 2001).

What drives translocation across the TOM complex? For matrix-targeted proteins it seems conceivable that the translocation is driven by sequential interactions of a presequence with different modules of the TOM complex to which it binds with increasing affinities (Komiya *et al.*, 1998; Mayer *et al*., 1995). However, movement past the *trans* binding site and the complete translocation across the TOM complex is only possible in the presence of the TIM23 complex and the membrane potential. The situation is somehow similar with hydrophobic inner membrane proteins of the carrier family. They appear to be in contact with the soluble "small" Tim complex in the IMS while other parts of the protein are still outside of mitochondria showing at least partial translocation across the outer membrane (Wiedemann *et al.*, 2001). However, complete translocation requires both the membrane potential and the TIM22 complex. In the case of small proteins of the IMS translocation across the TOM complex seems to be accompanied by the stable folding of the polypeptide chain in the intermembrane space, in most cases helped by the incorporation of a cofactor (Lutz *et al*., 2003; Nargang *et al*., 1988).

1.5. Sorting of β-barrel outer membrane proteins

For a long time it was believed that the TOM translocase is the only complex in the outer membrane involved in the biogenesis of mitochondrial proteins and that it alone is sufficient for translocation and sorting of all outer membrane proteins. Very recently it was shown that the biogenesis of β-barrel proteins in the outer membrane, such as porin and Tom40, requires an additional complex. This complex, named TOB (topogenesis of mitochondrial outer membrane β-barrel proteins) or SAM (sorting and assembly machinery), is specifically needed for the correct sorting and assembly of β-barrel proteins following translocation by the TOM complex (Paschen *et al*., 2003; Wiedemann *et al*., 2003). The TOB complex has a molecular mass of ca. 250 kDa and apparently consists of two proteins, Mas37 and

Tob55/Sam50 (Kozjak *et al*., 2003; Paschen *et al*., 2003). Tob55 is encoded by an essential gene indicating the importance of this complex for the biogenesis of mitochondria.

1.6. TIM23 translocase

All presequence containing preproteins emerging from the TOM complex are directed to the TIM23 complex which translocates them in a membrane-potential and ATP-dependent manner across the inner membrane (Figure 3). The TIM23 translocase can be divided into two functional parts: the membrane embedded translocation channel and the import motor at the matrix face. The membrane integrated part of the translocase is made up by two proteins, Tim17 and Tim23. Both proteins are integrated in the inner membrane by probably four transmembrane helices (Emtage and Jensen, 1993; Ryan *et al*., 1994). Tim23 has an additional ca. 100 amino acids long domain in the IMS. Hydrophobic segments of Tim17 and Tim23 are homologous but they cannot substitute for each other as these parts cannot be exchanged and both proteins are essential for cell viability (Ryan *et al*., 1998). When mitochondria are solubilized with digitonin, Tim17 and Tim23 form a stable ca. 90 kDa complex detected on the BN-PAGE (Dekker *et al*., 1997). If the separation is performed on a gel filtration column, Tim17 and Tim23 are found in a complex of the apparent molecular mass of ca. 280 kDa (Berthold *et al*., 1995).

The N-terminal domain of Tim23 acts as a receptor for precursors emerging from the TOM complex (Bauer *et al*., 1996). Furthermore, this domain is able to dimerize in a membrane-potential dependent manner and was therefore proposed to be a voltage sensor in the TIM23 complex. The Tim23 dimer is disrupted in the presence of a translocating chain and this probably leads to the opening of the translocation channel in the inner membrane (Bauer *et al.*, 1996). Recently it has been shown that the first fifty amino acids of Tim23 are tethered into the outer membrane (Donzeau *et al*., 2000). This two-membrane-spanning topology of Tim23 is thought to facilitate the transfer of precursor proteins from the TOM to the TIM23 complex by keeping the two membranes in proximity. It should be noted that a contact between Tim23 and the TOM complex has not been observed and that the proposed function of the first ca. 50 amino acids of Tim23 has recently been questioned (Chacinska *et al*., 2003).

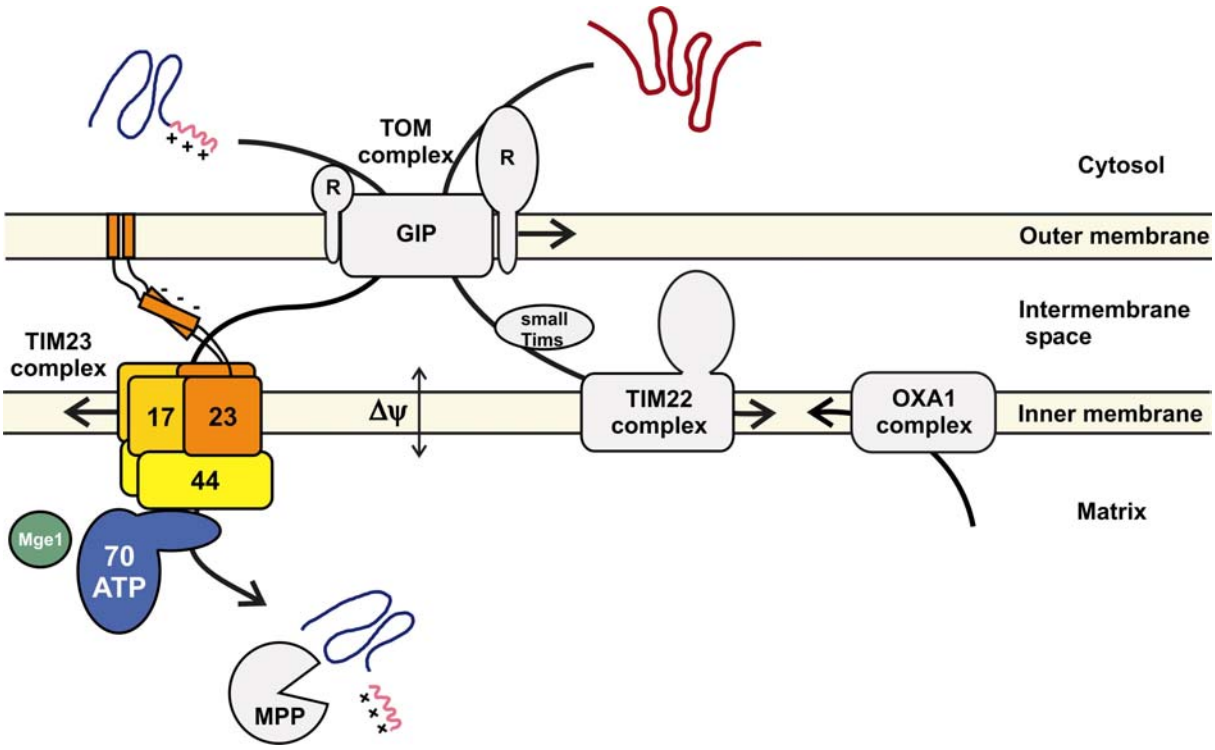

Figure 3. The presequence translocase of the inner membrane (TIM23 complex)

The TIM23 translocase enables the transport of presequence-containing preproteins across the inner membrane. It consists of the membrane embedded translocation channel and the import motor which is associated from the matrix side. The translocation channel is probably formed by Tim17 and Tim23. The first ca. 50 amino acids of Tim23 are tethered in the outer membrane and the next ca. 50 act as a presequence receptor and have the ability to dimerize in the ΔΨ dependent manner. The import motor consists of Tim44, mtHsp70 and Mge1. Tim44 binds both to the translocation channel and to the mtHsp70 thereby recruiting the chaperone to the import site. The import motor completes the translocation of preproteins into the matrix in the ATP-dependent cycles of mtHsp70 helped by Tim44 and the nucleotide exchange factor, Mge1.

Recombinant Tim23 expressed in *E. coli* is able, after refolding, to form cation-selective and presequence-sensitive channels in planar bilayers (Truscott *et al.*, 2001). The channel is formed by the membrane-embedded C-terminal half of the protein while the N-terminal half is needed for the selectivity and high sensitivity towards presequences (Truscott *et al.*, 2001). Whether the translocation channel *in vivo* is made up by Tim23 alone or Tim17 contributes to its formation remains to be shown.

Whereas some precursors can be integrated into the inner membrane without its help, complete translocation into the matrix is absolutely dependent on the functional import motor. The import motor is attached to the translocation channel at the matrix side of the inner

membrane and it consists of three known components – Tim44, mtHsp70 and Mge1. MtHsp70 is the central component of the import motor. It binds preproteins emerging from the TIM23 channel in a reaction cycle driven by ATP binding and hydrolysis and modulated by the other two proteins. The net result is the complete translocation of the preprotein into the matrix. All three proteins are encoded by essential genes showing the importance of this molecular machine for the cell (Craig *et al*., 1987; Ikeda *et al*., 1994; Maarse *et al*., 1992). What is known about its function?

In yeast, Tim44 is a peripheral membrane protein which can be released from the membrane by treatment with high concentrations of salt (Berthold *et al*., 1995; Moro *et al*., 1999). Upon solubilization of mitochondria with digitonin, Tim44 was found associated with the Tim17-Tim23 subcomplex in a salt-dependent manner (Moro *et al*., 1999). Human and rat Tim44, on the other hand, were found mostly soluble in the matrix (Bauer *et al*., 1999a; Ishihara and Mihara, 1998). Furthermore, no interaction of Tim44 to the Tim17-Tim23 subcomplex was observed in human or rat mitochondria (Bauer *et al*., 1999a; Ishihara and Mihara, 1998). It should be noted that this could be due to the high salt conditions used under which even yeast Tim44 mostly dissociates from the Tim17-Tim23 subcomplex. The binding site in the inner membrane for Tim44 is not known. Depletion of either Tim17 or Tim23 did not lead to the dissociation of Tim44 from the membrane possibly implying the presence of another, so far unknown component which recruits Tim44 to the membrane (Milisav *et al*., 2001). Yeast Tim44, recombinantly expressed and purified from *E. coli*, was shown to bind to cardiolipin-containing liposomes raising the possibility that the same kind of interaction occurs in mitochondria (Weiss *et al*., 1999). It is however difficult to envisage that the sole binding to membrane lipids could lead to the specific association with the Tim17-Tim23 subcomplex. Tim44 recruits mtHsp70 to the translocation channel (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994). These two proteins are found in an ATP-dependent subcomplex stable in Triton X-100. Addition of ATP in the solubilization buffer dissociates the complex (Horst *et al*., 1996; Schneider *et al*., 1994; von Ahsen *et al*., 1995). However if ATP was depleted from mitochondria prior to solubilization, no interaction of Tim44 to mtHsp70 was observed suggesting that Tim44 binds to the ATP form of mtHsp70 (Schneider *et al*., 1994).

MtHsp70, as all chaperones of the Hsp70 family, consists of the N-terminal nucleotide binding domain followed by the C-terminal peptide binding domain (Bukau and Horwich, 1998). Even though no member of the Hsp70 family has been crystallized so far, crystals of both domains were obtained separately and their structures solved (Flaherty *et al*., 1990; Zhu *et al*., 1996). The peptide binding domain consists of two subdomains (Zhu *et al*., 1996). The

first subdomain is made up by eight antiparallel β–strands which form a hydrophobic, substrate binding pocket and is followed by the second, α-helical subdomain which forms a lid-like structure needed to lock the peptide in the binding pocket. ATP hydrolysis on to the nucleotide binding domain is used to drive the conformational change in the peptide binding domain that alters its affinity for substrates (Bukau and Horwich, 1998). When ATP is bound to the nucleotide binding domain, the substrate binding pocket is in the open conformation and substrates are easily bound but also released. In the ADP form, the substrate binding pocket is in the closed state. Substrates can hardly enter it whereas those already bound are tightly held and are hardly released. This ATP-hydrolysis driven cycle allows members of the Hsp70 family chaperones to associate with substrate proteins in the ATP form and then hydrolyze ATP to bind them tightly in the ADP form. Release of ADP and rebinding of ATP finally triggers the release of the bound substrate. Both the ATP hydrolysis and the nucleotide exchange reactions are regulated by cochaperones. The ATPase activity of Hsp70s is stimulated by the members of the DnaJ protein family (Kelley, 1998). This is a heterogeneous family of proteins defined by a highly conserved ca. 80 amino acids long domain, the J-domain which is named after the first identified member, *E. coli* DnaJ. The J-domain is essential for the stimulation of the ATPase activity of Hsp70. In particular, an absolutely conserved motif consisting of three amino acids (HPD) is necessary for this stimulatory activity (Wall *et al*., 1994). NMR structures of two different J-domains showed that they comprise four helices with the HPD motif located in the loop between helices two and three (Qian *et al*., 1996; Szyperski *et al*., 1994). The nucleotide exchange reaction is stimulated by members of the GrpE protein family (Liberek *et al*., 1991). Members of this family drastically decrease the affinity of Hsp70 for ADP thus enabling its release and rebinding of ATP.

The mitochondrial import motor drives the translocation of preproteins into the matrix in an ATP-dependent cycle. Members of Hsp70 chaperone and GrpE co-chaperone families exist in the mitochondrial import motor - mtHsp70 and Mge1, respectively. However no member of the DnaJ family has been found. Interestingly, three J-domain proteins have been described in mitochondria but none of them is involved in the import process (Kim *et al*., 2001; Rowley *et al.*, 1994; Westermann and Neupert, 1997). The apparent lack of a J-protein in the import motor and a very limited sequence similarity between Tim44 and J-proteins have lead to the hypothesis that Tim44 is the J-like protein (Merlin *et al*., 1999; Rassow *et al*., 1994). However, there is no evidence for a significant homology of Tim44 to the J-domain proteins and, most importantly, Tim44 does not contain the absolutely conserved HPD motif making this hypothesis highly unlikely.

The actual mechanism of the mitochondrial import motor is not known. Two models were proposed. According to the Brownian ratchet or trapping model, Tim44 binds to the peptide binding domain of mtHsp70 and thereby recruits it to the outlet of the translocation pore. This allows mtHsp70 to bind to the incoming polypeptide chain as soon as it emerges from the channel. In this way backsliding of even short segments is prevented. MtHsp70 represents a trap which blocks the retrograde movement and spontaneous Brownian forward movement can be transduced into the vectorial transport by repeated cycles of mtHsp70 binding and release (Gaume *et al*., 1998; Neupert and Brunner, 2002; Okamoto *et al*., 2002). According to the power-stroke or pulling model, mtHsp70 is bound via its ATPase domain to Tim44 and actively pulls on the translocating chain. The force needed to pull in the polypeptide chain is generated by ATP-hydrolysis driven conformational changes of mtHsp70 (Matouschek *et al*., 1997; Matouschek *et al*., 2000; Voisine *et al*., 1999). Determination of the actual binding site for Tim44 on mtHsp70 could help to distinguish between the two models (Krimmer *et al*., 2000; Moro *et al*., 2002; Strub *et al*., 2002). The exact sequence of events within one cycle of the import motor reconstituted with purified components will bring new evidences for one or the other model. Such experiments have been initiated (Liu *et al*., 2003). It is, however, essential that no component is overlooked as a missing one could lead to misconception. Therefore purification and reconstitution of the complete TIM23 translocase is necessary.

1.7. TIM22 translocase

Transport of members of the mitochondrial carrier family which have six membrane spanning segments is a particularly demanding task for the cell. These hydrophobic stretches have to be shielded from the aqueous environments in the cytosol and in the intermembrane space and also inserted into the right membrane. Except for the use of the TOM complex, carrier proteins follow a completely different import route. While presequence-containing preproteins are translocated in a linear fashion, carrier proteins cross the outer membrane in loop-like structures (Endres *et al*., 1999; Wiedemann *et al*., 2001). In the IMS, they are recognized by a soluble complex of “small” Tims. This complex guides the carrier proteins to the TIM22 translocase which inserts them into the inner membrane (Figure 4).

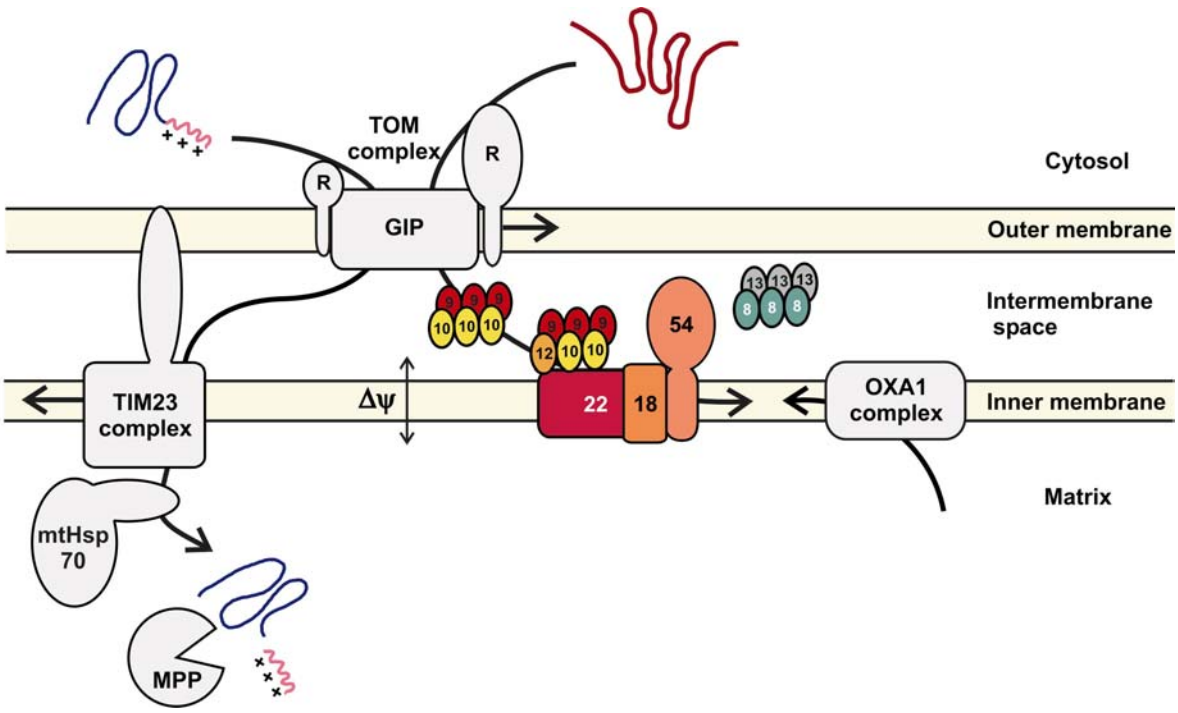

Figure 4. The carrier translocase of the inner membrane (TIM22 complex)

Hydrophobic inner membrane proteins of the carrier family cross the outer membrane in loop-like structures. They are recognized by the soluble complex of "small" Tim proteins in the IMS. There are two of these complexes: the Tim9-Tim10 complex which is involved in the translocation of the majority of precursors and the Tim8-Tim13 complex which has a more specialized role in the transport of some precursors. By binding to Tim12, soluble "small" Tim complexes deliver precursors to the TIM22 translocase which inserts them into the inner membrane in the ΔΨ-dependent reaction. The TIM22 translocase consists of three integral membrane proteins: the channel forming subunit, Tim22, and two subunits of so far not established functions, Tim18 and Tim54. A fraction of Tim9 and Tim10 is bound to the membrane integrated part of the translocase via Tim12.

The family of "small" Tim proteins consists of five homologous proteins: Tim8, Tim9, Tim10, Tim12 and Tim13 (Koehler, 2004; Paschen and Neupert, 2001; Rehling *et al*., 2003b). They share a conserved "twin CX_3C" motif in which two cysteines are separated by three amino acids and one such block is separated from the second one by 11-16 amino acids. This motif was originally proposed to bind zinc. Indeed, recombinantly expressed "small" Tim proteins have zinc bound (Rothbauer *et al*., 2001; Sirrenberg *et al*., 1998). In contrast, no zinc was found in proteins purified from yeast IMS and four cysteines rather appeared to form disulphide bonds (Curran *et al*., 2002a; Curran *et al*., 2002b). However, some recent experiments suggest that zinc binding in the IMS is needed for efficient import and stable

folding of "small" Tims (Lutz *et al*., 2003). Whether the disulphide bonds observed are just the artifact of the purification procedure or they do form *in vivo* transiently or under specific conditions is difficult to distinguish. It is possible that disulphide bond formation is actually a means to regulate the activity of "small" Tims and therefore the import process of carrier proteins.

Tim12 is the only "small" Tim protein stably associated with the inner membrane. All others are found mostly soluble in the IMS. Tim9 and Tim10 make a soluble, ca. 70 kDa complex composed of three copies of each subunit (Curran *et al*., 2002a). This complex has a general role in the import of all carrier proteins tested. Peptide scan experiments have shown that the Tim9-10 complex binds to the hydrophobic stretches of the ATP/ADP carrier (AAC) (Curran *et al*., 2002a). Small amounts of Tim9-10 complex can associate with the membrane embedded part of the TIM22 translocase in a reaction which is probably mediated by Tim12 (Adam *et al.*, 1999). Tim8 and Tim13 form a different ca. 70 kDa complex which consists of three Tim8 and three Tim13 subunits (Curran *et al*., 2002b). The Tim8-13 complex appears to have a more specialized role in the import. The precursor of Tim23 is integrated into the inner membrane by the TIM22 translocase but, unlike carriers, uses the Tim8-13 complex to pass the IMS (Leuenberger *et al*., 1999; Paschen *et al*., 2000). Whether Tim23 is the only substrate of this complex is not known. Peptide scan experiments have shown that Tim8-13 complex binds both the N-terminal hydrophilic part of Tim23 as well as the four hydrophobic segments (Curran *et al*., 2002b). No binding to the AAC was observed, in agreement with the finding that import of carriers does not depend on the Tim8-13 complex.

Tim9, Tim10 and Tim12 are encoded by essential genes while Tim8 and Tim13 are dispensable for yeast cell viability (Adam *et al*., 1999; Koehler *et al*., 1998a; Koehler *et al*., 1999a; Koehler *et al*., 1998b; Sirrenberg *et al*., 1998). However, the latter two are of particular medical importance as loss-of-function mutations in DDP1, the human homologue of Tim8, results in a severe neurodegenerative disease – the Mohr-Tranebjaerg syndrome (Bauer *et al*., 1999b; Koehler *et al*., 1999a).

The membrane embedded TIM22 translocase has a molecular mass of ca. 300 kDa and mediates insertion of carrier proteins into the inner membrane in a membrane potential driven reaction (Koehler *et al*., 1999b; Paschen and Neupert, 2001; Rehling *et al*., 2003b). The TIM22 complex consists of three integral membrane proteins: Tim22, Tim54 and Tim18, and, from the IMS side associated Tim9-Tim10-Tim12 subcomplex. Tim12 probably serves as a docking point for this subcomplex. It is not known via which component Tim12 is bound to the membrane integrated part of the complex. Tim18, although a stable constituent of the

complex, is most likely not directly involved in the insertion process itself. It is encoded by a non-essential gene and is proposed to function in the assembly and stabilization of the TIM22 complex (Kerscher *et al*., 2000; Koehler *et al*., 2000). Tim54 is anchored into the inner membrane by a single transmembrane domain and exposes a large domain into the IMS. Although initially found to be essential, a gentler way of deletion revealed that yeast can live without Tim54 (Kerscher *et al*., 1997; Kovermann *et al*., 2002). Tim54-depleted cells, however, grow very poorly suggesting an important role of Tim54 in the import process. Tim22 is the central component of the complex and the only really essential membrane embedded subunit. It was originally identified as a protein homologous to Tim17 and Tim23 but was present in a separate complex which turned out to be involved in the import of carrier proteins (Sirrenberg *et al*., 1996). Recombinantly expressed and purified from *E. coli*, Tim22 forms voltage-activated channels which respond to an internal targeting signal but not to a presequence (Kovermann *et al*., 2002). Electrophysiological measurements done with the purified TIM22 translocase revealed channels with similar basic characteristics but which, in contrast to the purified Tim22 alone, behaved like two tightly coupled pores (Rehling *et al*., 2003a). Indeed, single particle analysis of the purified complex showed the presence of particles with two stain-filled centers which may correspond to the two coupled pores observed in electrophysiological experiments (Rehling *et al.*, 2003a). The apparently opened channels of the TIM22 complex would be deleterious to the maintenance of the membrane potential across the inner membrane, so it is hard to believe that these channels are constitutively opened in mitochondria. Further studies will have to clarify the regulation of the pore opening. Other points which await further clarification are the actual mechanism of insertion of transmembrane segments into the inner membrane and the role of the membrane potential in the process.

1.8. Oxa1 translocase

TIM23 and TIM22 translocases mediate translocation across and insertion into the inner membrane of precursors which are coming from the IMS side of the inner membrane. Proteins encoded in the mitochondrial genome are appearing from the other side of the membrane and their insertion is mediated by neither of these two translocases but by the third one – the Oxa1 complex. The major and probably the only component of this complex is the Oxa1 protein itself (Hell *et al*., 1998; Nargang *et al*., 2002). Oxa1 is the founding member of a large family of highly conserved proteins whose members are not only present in mitochondria but also in

archaea, bacteria and thylakoid membranes of chloroplasts (Herrmann and Neupert, 2003; Yen *et al*., 2001). Members of this family share a conserved central core which consists of five transmembrane segments of similar lengths and spacing. This core domain is flanked by less conserved tails of various lengths. Recent evidence from bacterial and chloroplasts studies suggest that all Oxa1 homologues are involved in protein translocation (Kuhn *et al*., 2003).

Crosslinking experiments have demonstrated that Oxa1 is in direct contact with mitochondrially encoded precursors of the subunits of the respiratory chain complexes (Hell *et al*., 1998). This interaction is transient and detected only during their cotranslational insertion. This insertion is facilitated by an interaction of the matrix exposed C-terminal domain of Oxa1 with mitochondrial ribosomes (Jia *et al*., 2003; Szyrach *et al*., 2003). In addition to the mitochondrially encoded proteins, the Oxa1 complex inserts conservatively sorted proteins such as Oxa1 itself and subunit 9 of the *N. crassa* ATP synthase (Hell *et al*., 1998).

Oxa1 function is necessary for the respiratory activity of mitochondria and its deletion is therefore lethal for obligate aerobic organisms like *N. crassa* but can be tolerated on fermentable carbon sources in *S. cerevisiae*.

1.9. Aim of the present study

The aim of this study was to better understand the structure and the function of the TIM23 translocase. It was particulary important to see if any component had been missed so far. To do so, it was planned to identify the *tim23* gene in *Neurospora crassa* and to purify the TIM23 complex from this organism. Therefore, an *N. crassa* strain expressing a tagged version of the Tim23 protein was to be made and the purification procedure established. The purified complex was to be characterized in terms of the composition and the stability. Especially, any so far unknown component of the complex was to be analyzed thoroughly.

2. MATERIAL AND METHODS

2.1. Molecular biology methods

(Ausubel, 1991; Sambrook, 1989)

2.1.1. Isolation of plasmid DNA from *E. coli*

(Birnboim and Doly, 1979)

Plasmid DNA from *E. coli* was isolated with the "JetStar" kit (Genomed) using an anion-exchange column. Desired *E. coli* clones were grown overnight in 50 ml LB-Amp medium (Luria-Bertani medium: 10 g/l bacto-trypton, 5 g/l bacto-yeast extract, 5 g/l NaCl; supplemented with 100 mg/l ampicillin) at 37°C with vigorous shaking. Bacteria, pelleted by centrifugation for 5 min at 5000 rpm in JA-20 rotor (Beckman), were resuspended in 4 ml buffer E1 (50 mM TRIS/HCl, pH 8.0, 10 mM EDTA) and lysed with 4 ml buffer E2 (200 mM NaOH, 1% (w/v) SDS) for 5 min at RT. Cell-lysate was neutralized with 4.4 ml buffer E3 (3.2 M K-acetate, pH 5.5) and thus precipitated material removed by centrifugation for 10 min at 15000 rpm in JA-20 rotor (Beckman). In the mean time, the ion-exchange column was equilibrated with 10 ml buffer E4 (0.6 M NaCl, 100 mM Na-acetate, pH 5.0, 0.15% (w/v) Triton X-100). Clear supernatant, containing plasmid DNA, was loaded on the equilibrated column. After a washing step with 20 ml buffer E5 (0.8 M NaCl, 100 mM Na-acetate, pH 5.0), bound plasmid DNA was eluted with 5 ml buffer E6 (1.25 M NaCl, 100 mM TRIS/HCl, pH 8.5). DNA was precipitated by addition of 3.5 ml isopropanol and collected by centrifugation for 30 min at 9000 rpm in JA-20 rotor (Beckman). After a washing step with ice-cold 70% ethanol, DNA pellet was air-dried and finally resuspended in 150 µl sterile H_2O.

2.1.2. Amplification of DNA fragments by polymerase chain reaction (PCR)

PCR enables rapid and specific amplification of DNA fragments. A typical 50 µl reaction mix contained 5 µl supplied 10x PCR buffer, 2 µl dNTPs mix (each 10 mM), 6.25 µl each primer (20 pmol/µl) and 1 U of *Taq* polymerase. If the fragment was to be cloned, 0.5 U of *Pfu* polymerase was added in addition. Depending on the experiment, 10 ng plasmid DNA, 100 ng genomic DNA or 1 µl of the cDNA library was used as a template. When

cloning was checked by PCR, single *E.coli* colonies were resuspended in 15 µl sterile H_2O and 1 µl was used as a template for test PCR.
A typical program for PCR cycler is given below:

1.	95°C, 3 min		Inactivation of nucleases and denaturation of DNA template
2.	30 cycles:	95°C, 30 s	Denaturation
		55°C, 30 s	Primer annealing
		72°C, 1 min per 1 kb	Primer extension
3.	72°C, 10 min		Final extension step
4.	cooling to 4°C		

When PCR was done with degenerated primers the following program was used:

1.	94°C, 2 min	
2.	5 cycles:	94°C, 1 min
		50°C, 3 min
		72°C, 30 s
3.	30 cycles:	94°C, 1 min
		50°C, 1 min
		72°C, 30 s
4.	72°C, 10 min	
5.	cooling to 4°C	

The PCR product was analyzed by agarose gel electrophoresis (see 2.1.4.1.).

2.1.3. Enzymatic modifications of DNA

2.1.3.1. Restriction digestion of DNA

Restriction endonucleases recognize and cut specific DNA sequences. DNA to be digested was diluted in the buffer specific for the restriction enzyme used and the mixture supplemented with 2-4 U of enzyme per µg DNA. The digestion was done for 3 h at 37°C. Digested DNA was afterwards purified on a preparative agarose gel (see 2.1.4.1.).

2.1.3.2. Ligation of DNA fragments with T4 ligase

A DNA fragment (after restriction digestion) and a cloning vector (digested with the same or compatible enzymes) were ligated together with the ligase from bacteriophage T4. In a total volume of 10 µl, 100-200 ng linearized vector was mixed with 3-10 molar excess of DNA fragment in the ligation buffer (50 mM TRIS/HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 5% (w/v) PEG 8000). One unit of the T4 ligase was added and the mixture incubated overnight at 14°C. Ligation mixture was transformed into electrocompetent *E. coli* cells (see 2.1.5.)

2.1.4. Purification and analysis of DNA

2.1.4.1. Gel-electrophoresis of DNA

Horizontal agarose gel electrophoresis was used for separation of DNA fragments according to their sizes. Agarose was prepared in TAE buffer (40 mM TRIS/acetate, pH 7.5, 20 mM Na-acetate, 1 mM EDTA), mixed with 0.5 µg/ml ethidium-bromide and, while still hot, poured into precasted molds where it can solidify. DNA-containing solution was mixed with 1/5 volume of 5x loading dye (30% (v/v) glycerol, 0.25% (w/v) bromphenol-blue, 0.25% (w/v) xylencyanol) and loaded on an 0.8-3% (w/v) agarose gel, depending on the size of DNA fragments to be separated. Gels were run in TAE buffer at 60-150 V depending on the size. Separated DNA fragments were visualized under UV light.

2.1.4.2. Isolation of DNA from agarose gels

A piece of agarose containing the desired DNA fragment was cut out under UV light (366 nm). DNA was eluted from the gel with the "QIAquick Gel Extraction Kit" (Qiagen). Three volumes of QG buffer were added to the Eppendorf cup containing the agarose piece and the mixture incubated for 10 min at 50°C. When the agarose was completely dissolved, solution was loaded on the provided silica column to which DNA binds. The column was then washed with 750 µl of PE buffer and DNA finally eluted with 30 µl sterile H_2O. One µl of the eluted DNA was loaded on an analytical agarose gel to check the efficiency of purification and recovery.

2.1.4.3. Determination of DNA concentration

DNA concentration was determined by measuring the absorption of the DNA containing solution at 260 nm. One optical unit corresponds to 50 µg/ml of double stranded DNA.

2.1.5. Transformation of *E. coli* with plasmid DNA

(Dower *et al*., 1988)

Plasmid DNA or a ligation mixture was transformed into electrocompetent *E. coli* cells. Strains used were MH1 and XL1 Blue. Electrocompetent cells were made according to the following protocol. One ml of an *E. coli* overnight culture in LB medium was diluted into 500 ml LB medium and grown at 37°C with vigorous shaking until OD_{600} ca. 0.5. The culture was left on ice for 30 min and the cells then collected in sterile JA-10 tubes by centrifugation for 5 min at 5000 rpm at 4°C. Cells were washed with 500, 250 and finally 50 ml of sterile, ice-cold 10% glycerol. Final cell pellet was resuspended in 500 µl 10% glycerol and frozen at -70°C in 40 µl aliquots.

Electroporation was done with the "Gene Pulser" (Bio-Rad). The instrument was set at 2.5 kV, 400 Ω, 25 µF, time constant 8-9 ms. An aliquot of the electrocompetent cells was thawed on ice and mixed with 1 µl ligation mixture or plasmid DNA (ca. 10 ng). Mixture was transferred into a pre-cooled 0.2 cm electroporation cuvette and electrical pulse delivered at once. After the electroporation, cells were immediately diluted with 1 ml LB medium and shaken for ca. 30 min at 37°C. Cells were briefly spun, most of the medium poured off, cell pellet resuspended in the ca. 100 µl remaining medium and plated on LB-Amp plates (LB with 2% (w/v) agar supplemented with 100 µg/ml ampicillin). Plates were incubated overnight at 37°C. Usually, 18 clones were checked by test PCR (see 2.1.2.).

2.1.6. Plasmids used and cloning strategies

2.1.6.1. Overview of used plasmids

Plasmid	Reference
pGEM4-Tim23	This thesis
pMALcRI-Tim23(1-106)	This thesis
pCB1179-promTim23his	This thesis
pCB1179-promTim23hisflank	This thesis
pCB1179-promHisTim23flank	This thesis
pCB1532-promHisTim23flank	This thesis
pGEM4-Tim50	This thesis
pQE30-Tim50(189-540)	This thesis
pKS-bar-cpc1-Tim50his	This thesis
pGEM4-Tim14	This thesis
pQE30-Tim14(50-168)	This thesis
Constructs used for transcription/translation	
Su9(1-69)DHFR (N.c)	(Pfanner *et al.*, 1987b)
pcytb_2Δ19(167)DHFR (S.c)	(Schneider *et al.*, 1994)
pcytb_2Δ19(167)DHFR$_{K5}$ (S.c)	(Schneider *et al.*, 1994)
F1β (N.c)	(Rassow *et al.*, 1990)
CoxVa (S.c)	(Gartner *et al.*, 1995)
CoxVaΔ26-89 (S.c)	(Gartner *et al.*, 1995)
AAC (N.c)	(Pfanner *et al.*, 1987a)
Tim23 (S.c)	(Berthold *et al.*, 1995)
CCHL (S.c)	(Steiner *et al.*, 1995)

2.1.6.2. Cloning strategies

a) Screening of N. crassa cDNA library and gDNA with degenerate primers

T23.1.2	5´- GAT GAY YTI TGY TAY GGN AC -3´
T23.2.1	5´- GGI CCI TTY CTB GGN AA -3´

T23.2.2	5′- GGI CCI TTY TTR GGN AA -3′
T23.3.1	5′- RTC RTG YTT NCC NCG -3′
T23.3.2	5′- RTC RTG YTT NCC NCT -3′
T23.4	5′- AAC WSI ATY GCN GCN GG -3′
T23.5	5′- CAT IGG YTT NAR NCC -3′

Y=(C,T); B=(C,G,T); R=(A,G); N=(A,C,G,T); W=(A,T); S=(C,G)

In the first round of PCR, primers T23.1.2 (forward) and T23.5 (reverse) were used to amplify fragments from the cDNA library or gDNA as a template. PCR fragments of the right sizes were cut from the agarose gel, eluted and used as templates for nested PCRs with T23.2.1 or T23.2.2 as forward primers and T23.3.1 or T23.3.2 as reverse primers. Fragments of the right sizes were cut from the agarose gel, reamplified and immediately cloned into pCRII-TOPO TA cloning vector (Invitrogen) according to manufacturer´s instruction. Positive clones were identified by PCR and sent for sequencing.

b) Primers used to sequence cosmid pMOcosX #X2/E6 containing tim23

DM1	5′- CTT GGG GCT GGC TAG CAT CG -3′
DM2	5′- GCC CAT GAT GAT CTC GGG TGG -3′
DM3	5′- GGC TGT AAT GAA GAC C -3′
DM4	5′- TCT CTC CTC CCC GTG TGT GG -3′
DM7	5′- GCT ACA AGT GGC AGT AAC G -3′
DM8	5′- GTT GCG GGA GCC TCA TAC C -3′

c) Cloning of tim23 coding sequence into pGEM4

An intron-less sequence of the *N. crassa tim23* gene was amplified from the *N. crassa* cDNA library using primers EcoTIM23 and TIM23Bam. PCR fragment was cloned into pGEM4 vector using *Eco*RI and *Bam*HI restriction sites present in the primers.

EcoTIM23	5′- TTT GAA TTC ACA ATG TCC GGC CTT TGG AAC ACC -3′
TIM23Bam	5′- TTT GGA TCC TCA GTC AAC CTC GTT GGT CTG G -3′

d) Cloning of an N-terminal fragment of Tim23 (amino acids 1-106) into pMAL-cRI

The nucleotide sequence coding for amino acids 1 to 106 of *N. crassa* Tim23 was amplified from the plasmid pGEM4-Tim23 using primers EcoTIM23 and TIM23ABMal. PCR fragment was cloned into pMAL-cRI vector (New England Biolabs) using *Eco*RI and *Hind*III restriction sites incorporated into the primers.

EcoTIM23	5′- TTT GAA TTC ACA ATG TCC GGC CTT TGG AAC ACC -3′
TIM23ABMal	5′- GGG AAG CTT TTA GTC GTC AGT GAA GCC GCG CG -3′

e) Cloning of tim23 under its own promoter and with a C-terminal His-tag into pCB1179

The DNA segment containing 900 bp of sequence upstream of the *tim23* gene followed by the genomic *tim23* sequence was amplified from the cosmid pMOcosX #X2/E6 using primers EcoTIM23p and TIM238HBam. *Eco*RI and *Bam*HI restriction sites incorporated into the primers were used to clone the PCR product into pCB1179 vector. The reverse primer also enabled incorporation of 8 histidines at the C terminus of Tim23.

EcoTIM23p	5′- AAA GAA TTC TGA AGT GGA GGG GTT CGA TGG -3′
TIM238HBam	5′- TTT GGA TCC TCA ATG GTG GTG ATG GTG GTG ATG GTG GTC AAC CTC GTT GTT CTG GGG -3′

f) Cloning of 3′ flanking region of tim23 into pCB1179-EcopTim23hisBam

3′ flanking region (1kb) of the *tim23* gene was amplified from the cosmid pMOcosX #X2/E6 using primers BamTIM23-3 and TIM23Xba-3. PCR fragment was cloned at the 3′ end of the *tim23* construct in pCB1179 vector (see *e)*) using *Bam*HI and *Xba*I restriction sites from the primers.

BamTIM23-3	5′- AAA GGA TCC GGG GAC GAA GAA TGA AAT AGG -3′
TIM23Xba-3	5′- AAA TCT AGA GAT ATG GAT GGG AAA CTT GC -3′

g) Exchanging of the C-terminally tagged with the N-terminally His-tagged tim23 in pCB1179

The *tim23* promoter (900 bp sequence upstream of the *tim23* gene) was amplified from the cosmid pMOcosX #X2/E6 using primers HindTIM23p and TIM23Ecop. The same

template was used to amplify the coding sequence of the *tim23* using primers Eco9HTIM23 and TIM23Bam. Primer Eco9HTIM23 incorporated additional 30 nucleotides at the beginning of the *tim23* gene which code for a new start Met and a stretch of 9 histidines. After restriction digestion, these two PCR fragments were ligated in a single step into pCB1179-prom-Tim23His-flank (see *f)*) cut with *Hind*III and *BamH*I (which cut out the promoter and C-terminally His-tagged Tim23).

HindTIM23p	5′- CCC AAG CTT GAG CGA AGA GTG GTA AGT GCC -3′
TIM23Ecop	5′- AAA GAA TTC TGA AGT GGA GGG GTT CGA TGG -3′
Eco9HTIM23	5′- AAA GAA TTC ACA ATG CAC CAC CAT CAC CAC CAT CAC CAC CAT ATG TCC GGC CTT TGG AAC ACC -3′
TIM23Bam	5′- TTT GGA TCC TCA GTC AAC CTC GTT GGT CTG G -3′

h) Subcloning of promoter-9His-tim23-flank from pCB1179 into pCB1532

The DNA fragment coding for N-terminally His-tagged Tim23 with its promoter and 3′ flanking region was cut out with *Hind*III and *Xba*I from a construct in pCB1179 (see *g)*) and ligated into pCB1532 which was cut with the same restriction endonucleases.

i) Determination of the right start ATG in TIM50

First four primers from the table below contain four potential start ATGs of *tim50*. They were used in combination with the reverse primer Tim50Hind to amplify *tim50* in PCR reactions having *N. crassa* gDNA or the cDNA library as a template.

Tim50ATG1	5′- TTT TAA GCC ATA TTG GAG TTG AGA -3′
Tim50ATG2	5′- AAF ACG TGA TTG TCA TCC ATC C -3′
Tim50ATG3	5′- TCC CCC ACT GTC TTT GAC GC -3′
EcoTim50	5′- AGG GAA TTC ATG ATG CTG TCC AGA GCT GCC -3′
Tim50Hind	5′- ACC CAA GCT TAG GCC TTC TTC TCT GAC TCG -3′

j) Cloning of tim50 into pGEM4

An intron-less version of *tim50* gene was amplified from *N. crassa* cDNA library using primers EcoTim50 and Tim50Hind. PCR product was digested with *Eco*RI and *Hind*III

whose sites were incorporated into the primers and cloned into pGEM4 cut with the same enzymes.

EcoTim50	5′- AGG GAA TTC ATG ATG CTG TCC AGA GCT GCC -3′
Tim50Hind	5′- ACC CAA GCT TAG GCC TTC TTC TCT GAC TCG -3′

k) Cloning of Tim50(189-540) into pQE30

A nucleotide sequence coding for amino acids 189 to 540 of *N. crassa* Tim50 was amplified from pGEM4-Tim50 using primers KpnTim50C and Tim50Hind. PCR product was cloned into pQE30 vector using *Kpn*I and *Hind*III restriction sites from the primers.

KpnTim50C	5′- GGG GTA CCA TCT ACT TGG GAA GGG AAT GGG -3′
Tim50Hind	5′- ACC CAA GCT TAG GCC TTC TTC TCT GAC TCG -3′

l) Cloning of tim50-His into pKS-bar-cpc1

Genomic *tim50* sequence was amplified from *N. crassa* gDNA using primers EcoTim50 and Tim50HisCla. The latter primer incorporated additional 27 nucleotides coding for 9 histidines at the C terminus. Using *Eco*RI and *Cla*I restriction sites from the primers, obtained PCR product was cloned into pKS-bar-cpc1 vector.

EcoTim50	5′- AGG GAA TTC ATG ATG CTG TCC AGA GCT GCC -3′
Tim50HisCla	5′- CCA TCG ATT TAG TGA TGG TGGG TGA TGG TGG TGA TGG TGG GCC TTC TTC TCT GAC TCG -3′

m) Cloning of TIM14 into pGEM4

Coding sequence of *TIM14* gene was amplified from yeast gDNA using primers BamYLR008c_1 and YLR008cPst. Obtained PCR product was cloned into pGEM4 vector using *BamH*I and *Pst*I restriction sites in the primers.

BamYLR008c_1	5′- CCG GAT TCA TGA GTT CTC AAA GTA ATA CTG G -3′
YLR008cPst	5′- CCC TGC AGT TAT TTG CTA ATA CCC CTT TTT TCC -3′

n) Cloning of Tim14(50-168) into pQE30

A nucleotide sequence coding for amino acids 50 to 168 of Tim14 was amplified from yeast gDNA using primers BamYLR008c_2 and YLR008cPst. *BamH*I and *Pst*I restriction sites from the primers were used to clone the obtained PCR product into pQE30 vector.

BamYLR008c_2	5′- CCG GAT CCA TGG ACC TTT ATT TTG ATC AAG C -3′
YLR008cPst	5′- CCC TGC AGT TAT TTG CTA ATA CCC CTT TTT TCC -3′

2.1.7. Screening of *N. crassa* cosmid library

2.1.7.1. Preparation of a membrane containing pMOcosX #X cosmid library

pMOcosX #X (Orbach, 1994) cosmid library is supplied as a collection of 25 separate 96-well microtiter plates. As a first step, the whole library was amplified in duplicate on LB-Amp plates using a device which enables transfer of all 96 clones from one microtiter plate in a single step. One set of plates was used to isolate plasmid DNAs and the other was left at 4°C. Plasmid DNAs were isolated as follows. All 96 bacterial colonies from a single plate were collected into one JA-20 tube and further processed as a single midi-prep. In total 25 of these "collective" midi-preps were made, corresponding to 25 microtiter plates. Final DNA pellets were resuspended in 100 µl of 10 mM TRIS/HCl, pH 8.0. Two separate nylon membrane were then made by dot-blotting 1 µl of non-diluted and 1 µl of each 1:10 and 1:100 diluted "collective" plasmid DNAs. After short air-drying, membranes were placed between layers of 3MM Whatman paper and baked in an oven at 80°C for 1 h to fix the DNA. Membranes were then ready for probing with DIG-labeled probe (see 2.1.7.2.). A result was considered positive if the same dot gave positive signal on both membranes.

Incubation of these membranes enabled the identification of the microtiter plate containing the positive clone. In order to identify the exact clone, second set of plates, i.e. the ones identified as positives by hybridization, was used in a PCR screen. Bacteria were pooled in a way that each row and each column gave a separate template for PCR screen (8 rows and 12 columns, 20 PCRs in total). One row and one column as template give positive PCR result thus unambiguously identifying the clone containing the cosmid of interest.

2.1.7.2. Preparation of DIG-labeled probes

The hybridization probe for pMOcosX #X library screen was made using the "PCR DIG Probe Synthesis Kit" (Roche). This kit enables direct digoxigenin (DIG)-labeling of DNA fragments during PCR reaction by incorporation of DIG-dUTP. A typical labeling reaction contained 5 µl 10x PCR buffer with $MgCl_2$, 5 µl 10x PCR DIG probe synthesis mix (2 mM each dATP, dCTP, dGTP, 1.3 mM dTTP, 0.7 mM DIG-11-dUTP), 0.3 µl Enzyme mix (Expand High Fidelity), 1 µl each primer (20 pmol/µl), 1 µl template DNA and 37 µl H_2O. A control reaction was always done in parallel. It differed only in the nucleotide mix added – control reaction contained no DIG-11-dUTP. PCR was done as described in 2.1.2. Incorporation of labeled nucleotides was checked by loading a portion (5 µl) of each reaction on an agarose gel - the labeled PCR product migrates slower then the unlabeled, control PCR product due to the presence of DIG-labeled nucleotides.

2.1.7.3. Hybridization of Southern blots with probes

Nylon membrane was incubated for 2 h at 42°C in the prehybridization buffer (5x SSC, 50% formamid, 0.1% Na-laurylsarcosin, 0.02% SDS, 2% blocking reagent) and then hybridized overnight at 42°C with DIG-labeled probe in the prehybridization buffer (50 µl labeling PCR was diluted in 25 ml prehybridization buffer; before use, probe was boiled for 5 min at 95°C and quickly chilled on ice). Nonspecifically bound DNA was removed by two 5 min incubations at RT in the washing solution 1 (2x SSC, 0.1 % SDS) and then two 15 min incubations at 68°C in the washing solution 2 (0.5x SSC, 0.1% SDS). Membrane was briefly washed with buffer P1 (150 mM NaCl, 0.3% Tween 20, 100 mM maleic acid, pH 7.5) and incubated for 30 min in the blocking solution (1% (w/v) blocking reagent in P1). To detect DIG-labeled fragments, membrane was incubated for 30 min with antidigoxigenin antibodies labeled with alkaline phophatase, 1:10000 diluted in the blocking solution. Membrane was washed 2x 15 min with P1 and rinsed twice with P3 (100 mM NaCl, 100 mM TRIS/HCl, pH 9.5). After addition of a chemiluminescent substrate, CSPD, membrane was incubated for 5 min at RT and 15 min at 37°C and then exposed to an X-ray film.

Membrane was stripped by two incubations in 0.2 M NaOH, 1% SDS for 15 min at 37°C. After a brief washing in 2x SSC, membrane was either dried or could be incubated with a new probe.

2.2. Methods of *N. crassa* genetics

2.2.1. Overview of used *N. crassa* strains

Strain	Genotype	Origin or source
wt	A	Fungal Genetics Stock Center (FGSC) #2489 (74-OR23-1VA)
T23R4-12	Sheltered heterokaryon: (*acr-2 trp-1inl inv mei-2 tim23^{RIP}* + *ad-2 am132 inl inv mei-2*), mating type unknown.	(Mokranjac *et al*., 2003a)
HisTim23	*acr-2 trp-1 inl inv mei-2 tim23^{RIP}*, also contains an ectopic copy of *tim23* with an N-terminal nonahistidinyl tag	This thesis
Tim22His	A *bar*^{R}	C. Kozany, unpublished
OxaHis	*cyh-2 lys-2 leu-5 mei-2 oxa1RIP oxa1RIP* (EC), also contains an ectopic *oxa1* encoding a functional version of the protein with a C-terminal hexahistidinyl tag	(Nargang *et al*., 2002)
Tim50His	A *bar*^{R}	This thesis

2.2.2. Preparation of spheroplasts

(Vollmer and Yanofsky, 1986)

Conidia from a 7-10 days old culture grown on a solid medium were collected with sterile water and filtered through 3 layers of sterile cheesecloth. One hundred ml 1x Vogel´s medium (see 2.2.6.) was inoculated with 10 ml conidial suspension (ca. 1x10^{9} conidia) and vigorously shaken for ca. 2.5 h at 37°C or until most conidia germinated. From there on everything was done on ice. Conidia were pelleted by centrifugation (8 min, 4000 rpm, JA-20), washed once with 1 M sorbitol, resuspended in 10 ml 1 M sorbitol and finally transferred into a sterile flask. Twenty mg of Novozyme 234 (Sigma) was added and the flask gently shaken (80 rpm) at 25°C. Spheroplasting was checked after 30 min and

eventually continued for additional 30 min. Spheroplasts were gently poured into JA-20 tube, flask rinsed with 1 M sorbitol and the tube filled with 1 M sorbitol. Spheroplasts were pelleted (10 min, 2000 rpm, JA-20), washed twice with 30 ml 1 M sorbitol and once with 30 ml STC. Final pellet was resuspended in 7.5 ml STC. Two ml DMSO and 2 ml PTC were added and the suspension mixed gently but thoroughly. Spheroplasts were aliquoted (500 µl) and frozen at -70°C.

STC	1 M sorbitol, 50 mM TRIS/HCl, pH 8.0, 50 mM $CaCl_2$
PTC	40% (w/v) PEG 4000, 50 mM TRIS/HCl, pH 8.0, 50 mM $CaCl_2$

2.2.3. Transformation of spheroplasts

(Vollmer and Yanofsky, 1986)

Spheroplasts were thawed at RT. In a sterile Eppendorf cup 5 µl DNA (1-5 µg) was mixed with 2 µl spermidine and 5 µl heparin (5 mg/ml in STC) and the mixture incubated for 5 min on ice. 100 µl spheroplasts were added, mixed gently and incubated on ice. After 30 min, 1 ml PTC (at RT) was added, mixed thoroughly and incubated for 20 min at RT. Spheroplasts were then added to the top agar, mixed thoroughly and immediately poured onto bottom agar plates. After solidification of the top agar, plates were incubated at 30°C for ca. 2 days.

- Top agar is first completely melted at 95°C and then kept at 60°C. Just before addition of spheroplasts it is cooled to 54°C.
- Bottom agar plates are left for at least 1 h at 37°C before plating to prevent immediate solidification of the top agar.

a) Transformation of wild type N. crassa with constructs in pCB1179

This plasmid confers resistance to hygromycin. Hygromycin was added to bottom agar to a final concentration of 200 µg/ml.

b) Transformation of T23R4-12 with the construct in pCB1532

This plasmid confers resistance to chlorimuron ethyl. It was added at 200 µg/ml to the bottom agar. To immediately select for the transformation of the *tim23*^{RIP} nucleus, bottom agar also contained inositol (10 µg/ml) and tryptophan (100 µg/ml) as well as acriflavine (25 µg/ml), selection marker present in the *tim23*^{RIP} nucleus.

c) Transformation of wild type N. crassa with constructs in pKS-bar-cpc1

This plasmid confers resistance to BASTA. It was added to 3% (w/v) to bottom agar. Also, bottom and top agars contained 0.5% (w/v) prolin and Vogel´s salts without nitrogen.

10x FIGS	100 g sorbose, 2.5 g fructose, 2.5 g glucose, H_2O to 500 ml autoclave
Top agar	10 ml 50x Vogel´s, 91 g sorbitol, 14 g agar, H_2O to 450 ml autoclave, add 50 ml 10x FIGS, mix and make 8 ml aliquots in sterile 15 ml Falcon tubes
Bottom agar	10 ml 50x Vogel´s, 7.5 g agar, 440 ml H_2O autoclave, before pouring add 50 ml 10x FIGS and selective drugs

2.2.4. Selection of transformants

About 40 transformants were taken out from a selective plate using an inoculation needle and placed into 2 ml selective liquid media. After 3 days at 25°C, growing clones were amplified on slant agars and afterwards checked for the expression of His-tagged proteins (see 2.5.1.1.).

2.2.5. Generation of homokaryotic strains by microconidiation

N. crassa conidia usually contain 2 to 3 nuclei and in most cases only one of them is transformed. This means that the transformed nucleus is "contaminated" with the non-transformed ones and, if the culture is not constantly selected for the transformed one, it might be lost. Therefore, homokaryotic strains were generated by microconidiation (Ebbole, 1990). Heterokaryotic transformants were grown at RT with 12 hour light/dark cycles for ca. 10 days on iodoacetate-containing slant agars which leads to generation of mononuclear microconidia. Conidia were collected with 2 ml sterile water by vigorous vortexing for 1 min and passed through 5 µm filter (Millipore). All macroconidia are kept by the filter while smaller microconidia go through. Microconidia were then plated on selective sorbose plates and afterwards checked as described under 2.2.4.

Microconidiation medium	0.5% (w/v) sucrose, 0.1x SC-solution, 2% (w/v) agar after autoclaving add filter-sterilized iodoacetate (0.1 M) to 1 mM final; pour immediately 6 ml into sterile 16x160 test tubes and make slant agars
1 x SC-solution	1 g KNO_3, 0.7 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.1 g NaCl, 0.1 ml biotin solution, 0.1 ml trace elements solution and H_2O to 1 l, pH 6.5 (make fresh)
Sorbose plates	0.03% glucose, 2% Vogel´s, 1% sorbose, 2% agar autoclave 15 min at 110°C; after cooling down to ca. 60°C add a selective drug

2.2.6. Cultivation of *N. crassa*

N. crassa was grown essentially as described (Davis and De Serres, 1970). To obtain conidia, *N. crassa* was grown at 25°C under constant light on a solid full medium for ca. 7 days. Conidia were collected with sterile water and their number determined (1 OD_{420} corresponds to 2.6 x 10^8 conidia/ml). Liquid cultures were inoculated with 1 x 10^9 conidia/l and grown at 25°C in minimal medium under constant light and aeration for 16-24 h.

Trace elements stock solution	Dissolve salts sequentially in ca. 70 ml H_2O: 5 g citric acid x 1 H_2O, 5 g $ZnSO_4$ x 7 H_2O, 1 g $Fe(NH_4)_2(SO_4)_2$ x 6 H_2O, 0.25 g $CuSO_4$ x $5H_2O$, 0.05 g $MnSO_4$ x 1 H_2O, 0.05 g H_3BO_3, 0.05 g Na_2MoO_4 x 2 H_2O bring volume to 100 ml with H_2O; add 1 ml chloroform as preservative
Biotin stock solution	10 mg biotin in 100 ml 50% (v/v) ethanol; store at 4°C
50 x Vogel´s salts	To 750 ml H_2O add: 150 g Na_3-citrate x 5 H_2O, 250 g KH_2PO_4 (anhydr.), 100 g NH_4NO_3, 10 g $MgSO_4$ x 7 H_2O, 5 g $CaCl_2$ x 2 H_2O, 5 ml trace elements stock solution, 2.5 ml biotin stock

	solution bring volume to 1 l with H_2O; add 1 ml chloroform as preservative
Minimal medium	2% sucrose (or glucose), 1 x Vogel´s salts in H_2O for solid medium add 2% agar if necessary, add auxotrophic markers
Vitamin stock solution	10 mg vitamin B1, 5 mg vitamin B2, 5 mg vitamin B6, 5 mg aminobenzoic acid, 5 mg nicotine amide, 100 mg choline-hydrochloride, 1 mg folic acid, 100 mg inositol, 50 mg Ca-pantothenate, H_2O to 1 l
Full medium	20 ml 50 x Vogel´s salts, 10 ml glycerol, 10 g sucrose, 10 ml vitamin stock solution, 2.5 g yeast extract, 1 g casein hydrolysat, H_2O to 1 l add 0.5 g agar and 25 ml solution per flask

2.2.7. Labeling of *N. crassa* cells with ^{35}S-sulphate

N. crassa cells were metabolically labeled with ^{35}S-sulfate essentially as described in (Hennig and Neupert, 1981). Two l minimal medium was made with Vogel´s salts without sulfate and supplemented with 20 mg/l $MgSO_4$ and 0.5 ml $Na_2{}^{35}SO_4$ (2 mCi). Medium was inoculated with 2 x 10^9 wild type *N. crassa* conidia and the culture grown as described in 2.2.6.

2.3. Methods in *S. cerevisiae* genetics

2.3.1. Overview of used *S. cerevisiae* strains

Strain	**Reference**
YPH501	(Sikorski and Hieter, 1989)
YPH499	(Sikorski and Hieter, 1989)
W334	(Hovland *et al.*, 1989)
D273-10B	Deutsche Stamm Sammlung für Mikroorganismen
GAL-Tim17	(Milisav *et al*., 2001)

GAL-Tim23	(Milisav *et al*., 2001)
GAL-Tim44	(Milisav *et al*., 2001)
Tim14-HA	This thesis
TIM14/tim14:HIS3	This thesis
GAL-Tim14	This thesis
GAL-HisTim14	This thesis
mtHsp70His	(Kronidou *et al*., 1994)
Ssc1-3	(Gambill *et al*., 1993)

a) HA-tagging of Tim14

Tim14 was HA-tagged on the chromosome by homologous recombination of a PCR product in the diploid yeast strain YPH501. PCR product contained an auxotrofic-marker-cassette *klTRP1* and sequences homologous to the last 45 bp of *TIM14* gene and first 45 bp of its 3´ UTR. This construct was amplified from pYM3 plasmid (Knop *et al*., 1999) using primers YLR008c-S2 and YLR008c-S3 and transformed into yeast. Via the regions homologous to the *TIM14*, PCR product was stably integrated into the chromosome. To isolate positive clones, yeast transformants were grown on selective medium lacking tryptophan and homologous recombination checked by PCR.

YLR008-S3	5´- ATA AAC GAA GCT AAG GAC TTT TTG GAA AAA AGG GGT ATT AGC AAA CGT ACG CTG CAG GTC GAC -3’
YLR008-S2	5´- GCA TAT ATG CAA TTG CAA TAA CTC ATT TTA GGT TCC CGT TTT ACC ATC GAT GAA TTC GAG CTC G-3’

b) Deletion of TIM14 gene in diploid yeast strain YPH501

TIM14 gene was deleted by homologous recombination of a PCR product in the diploid yeast strain YPH501. PCR product contained an auxotrofic-marker-cassette *HIS3* and short sequences homologous to the flanking regions of the *TIM14* locus. Primers YLR008-delta-For and YLR008-S2 were used to amplify the construct from pFA-HIS3MX6 (Wach *et al*., 1997). Diploid yeast strain YPH501 was transformed, transformants selected on a selective medium lacking histidine and positive clones checked by PCR.

YLR008-delta-For	5'- CAC AGT TTA ATA AGG TTG CAT AAA CAC TTC CAC CCG CAC AAT ATC CGT ACG CTG CAG GTC GAC -3'
YLR008-S2	5´- GCA TAT ATG CAA TTG CAA TAA CTC ATT TTA GGT TCC CGT TTT ACC ATC GAT GAA TTC GAG CTC G-3'

c) Placing TIM14 under GAL10 promoter

PCR-based strategy was used to change the endogenous *TIM14* promoter with the *GAL10* one. PCR product containing an auxotrofic-marker-cassette *HIS3*, *GAL10* promoter and sequences homologous to the TIM14 promoter was amplified from the plasmid pTL26 (Lafontaine and Tollervey, 1996) using primers YLR008-downfor-145 and YLR008-downrevmut-0. After transformation into haploid yeast strain YPH499 and selection on selective medium lacking histidine, positive clones were checked by PCR.

YLR008-downfor-145	5'- TGA AAG CCA TTT GAA TCA ACG TAG GAC TCA CTC GAC GGA GAC CCT CTC TTG GCC TCC TCT AGT -3'
YLR008-downrevmut-0	5'- GTT GTG GTG CCT CAA TAG AAT TAC CAG TAT TAC TTT GAG AAC TCA TTA AGC CCG AAT TCC TTG AAT TTT CAA A -3'

d) Construction of N- terminally His-tagged Tim14 under GAL10 promoter

PCR-based strategy was used to exchange the endogenous promoter of *TIM14* with *GAL10* promoter and at the same time introduce a His-tag at the N-terminus of Tim14. PCR product was amplified from the plasmid pTL32 (Lafontaine and Tollervey, 1996) using primers YLR008-N8HIS and YLR008-downfor-145. Transformation and selection was done as under *c)*.

YLR008-N8HIS	5'- TTG TGG TGC CTC AAT AGA ATT ACC AGT ATT ACT TTG AGA ACT CAT ATG GTG ATG GTG ATG GTG ATG GTG AGA -3'
YLR008-downfor-145	5'- TGA AAG CCA TTT GAA TCA ACG TAG GAC TCA CTC GAC GGA GAC CCT CTC TTG GCC TCC TCT AGT -3'

2.3.2. Transformation of *S. cerevisiae* with recombinant DNA

Yeast was transformed using Li-acetate method according to (Gietz *et al*., 1992). A 50 ml yeast culture was grown until OD_{600} ca. 0.5 at 30°C with shaking. Cells were collected (3 min, 3000 rpm, Sigma centrifuge), washed once with 25 ml sterile H_2O and resuspended in 1 ml 100 mM Li-acetate. Cell suspension was transferred into a 1.5 ml Eppendorf cup, cells collected by centrifugation in a tabletop centrifuge and resuspended in 400 µl 100 mM Li-acetate. 50 µl of this suspension was used pro transformation. Cells were pelleted, supernatant removed and the cell pellet overlayed, in the following order, with 240 µl 50% (w/v) PEG 3550, 36 µl 1 M Li-acetate, 5 µl heat-denatured and quickly cooled salmon-sperm DNA (10 mg/ml), 60 µl H_2O and 5 µl DNA (0.1-10 µg plasmid DNA or PCR product). Mixture was vigorously vortexed for ca. 1 min, shaken at 30°C for 30 min and heat-shocked at 42°C for 20-25 min. Cells were pelleted by centrifugation (15 s, 6000 rpm, tabletop centrifuge), resuspended in 150 µl H_2O and plated on selective media.

2.3.3. Cultivation of *S. cerevisiae*

Yeast was normally grown on lactate medium or YPD at 30°C. Temperature-sensitive mutants were grown at 24°C. Liquid cultures were incubated on shakers (140 rpm). They were inoculated from agar plates or directly from glycerol stocks. For isolation of mitochondria, cells were passaged for ca. 3 days in the way that OD_{600} never exceeded 1. If mitochondria depleted of one its essential proteins were to be prepared, a yeast strain having the corresponding gene under *GAL* promoter was grown for ca. 3 days on galactose-containing media after which cells were collected, washed with water and resuspended in glucose-containing media. Cells were grown in glucose medium for the indicated time periods.

Lactate medium (supplemented with 0.1% glucose or galactose)	3 g yeast extract, 1g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2$ x 2 H_2O, 0.5g NaCl, 1.1 g $MgSO_4$ x 6 H_2O, 0.3 ml 1% $FeCl_3$, 22 ml 90% lactic acid, H_2O to 1 l, pH 5.5 (adjusted with KOH)

YPD medium	10 g yeast extract, 20 g bacto-pepton, 5.5 ml 90% lactic acid, H_2O to 930 ml pH 5.5 (adjusted with KOH) after autoclaving add 67 ml 30% glucose
YPGal medium	10 g yeast extract, 20 g bacto-pepton, 5.5 ml 90% lactic acid, H_2O to 930 ml pH 5.5 (adjusted with KOH) after autoclaving add 67 ml 30% galactose
SD medium	1.74 g yeast nitrogen base, 5 g ammonium sulfate, 20 g glucose, H_2O to 1 l

If corresponding plates were made, 2% (w/v) agar was added before autoclaving. For selective media, amino acids solutions (His, Leu, Lys, all 10 mg/ml) and uracil and adenine solutions (both 2 mg/ml) were separately autoclaved for 20 min at 120°C, with the exception of tryptophan (10 mg/ml) which was filter sterilized. They were added to the medium before pouring the plates.

2.4. Methods in protein biochemistry

2.4.1. Protein preparation

2.4.1.1. In vitro synthesis of mitochondrial preproteins

All mitochondrial preproteins were cloned into pGEM vectors under the control of *Sp6* promoter. To radioactively label them, translation reaction was done in the presence of ^{35}S-methionine.

a) In vitro Transcription

(modified after (Melton DA, 1984))

A typical transcription mix (100µl) contained 20 µl 5x transcription buffer (200 mM TRIS/HCl, pH 7.5, 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine), 10 µl 0.1 M DTT, 4 µl RNAsin (40 U/µl), 20 µl NTPs (10 mM each), 5.2 µl m^7G(5´)ppp(5´)G, 3 µl *Sp6* polymerase, 27 µl H_2O and 10 µl plasmid DNA. The reaction mix was incubated for 1 h at 37°C after which 10 µl 10 M LiCl and 300 µl ethanol were added. Reaction was cooled at - 20°C for 30 min and RNA precipitated by centrifugation for 20 min at 20000 rpm

(Sigma centrifuge). RNA pellet was washed with ice-cold 70% (v/v) ethanol, resuspended in 100 µl H_2O with 1 µl RNAsin and stored at -70°C.

b) In vitro Translation

(Pelham and Jackson, 1976)

A typical translation mix contained 50 µl rabbit reticulocyte lysate, 20 U RNAsin, 1.75 µl amino acid mix (all proteinogenic amino acids except methionine, 1mM), 6 µl ^{35}S-methionine, 3.5 µl 15 mM Mg-acetate and 12.5 µl RNA. After 1 h at 30°C, incorporation of labeled methionine was stopped by addition of 6µl 58 mM cold methionine. After addition of 12 µl sucrose (1.5 M), ribosomes and aggregated proteins were removed by centrifugation (30 min, 45000 rpm, TLA 45 rotor, 4°C). Lysate was aliquoted, frozen in liquid nitrogen and stored at - 70°C.

c) TNT Coupled reticulocyte lysate system

As an alternative to the two separate steps of transcription and translation, the TNT Coupled reticulocyte system offers a possibility to simultaneously perform both reactions. TNT reaction mixture (50µl) contained: 25µl TNT rabbit reticulocyte lysate (Promega), 2 µl TNT reaction buffer, 1 µl TNT *Sp6* RNA polymerase, 1 µl amino acid mix, 2 µl ^{35}S methionine (10 mCi/ml), 1 µl RNasin (40 U/µl), 2 µl DNA template (0.5 µg/µl). The reaction was incubated for 90 min at 30°. Aggregated proteins were removed by centrifugation (30 min, 45000 rpm, TLA 45 rotor, 4°C). Lysate was aliquoted, frozen in liquid nitrogen and stored at -70°C.

2.4.1.2. Purification of recombinant proteins with MBP-tag from E. coli

First 106 amino acids of *N. crassa* Tim23 fused to maltose binding protein (MBP) were expressed in *E.coli* from pMAL-cRI vector (New England Biolabs). Five ml of an overnight *E. coli* culture were diluted into 250 ml LB-Amp and grown until OD_{600} ca. 0.5 at 37°C. Expression of the recombinant protein was induced by 0.5 mM IPTG and the culture grown for additional 2 h at 37°C. Cells were pelleted by centrifugation for 10 min at 5000 rpm in JA-10 rotor and resuspended in 20 ml buffer A (20 mM HEPES/NaOH, pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 1mM PMSF). Lysozyme was added to 1 mg/ml and lysis done for 45 min at 4°C. Cells were completely broken by sonication (10x 12 s, Branson sonifier, setting 4, 80% duty cycle). After centrifugation for 15 min at 15000 rpm in JA-20 rotor, clear supernatant was loaded on a 7 ml Amylose-column (New England Biolabs) preequilibrated in buffer A. Column was washed with 35 ml of buffer A

and bound proteins eluted with 10 mM maltose in buffer A. Expression and purification were monitored by SDS-PAGE (see 2.4.3.1) and CBB staining (see 2.4.3.2.).

2.4.1.3. Purification of recombinant proteins with His-tag from E. coli

Hexahistidine tagged recombinant proteins were expressed in *E. coli* from pQE-30 or pQE-70 vectors (Qiagen). An *E. coli* strain containing the plasmid expressing the desired protein was inoculated in ca. 20 ml LB-Amp medium and grown overnight at 37°C. Five ml of the overnight culture was diluted into 250 ml LB-Amp and grown at 37°C until OD ca. 0.5. Expression of the His-tagged protein was induced with 1 mM IPTG. Cells were grown for additional 2-3 h. Finally, depending on its solubility, protein was purified on a NiNTA-agarose either under native or under denaturing conditions.

a) Purification under denaturing conditions

The whole procedure was done at RT. Bacterial cell pellet was resuspended in 25 ml buffer A (100 mM NaH_2PO_4, 10 mM TRIS, 6 M guanidium-HCl, pH 8.0) and gently stirred for ca. 30 min at RT, until the solution became translucent. Cell debrices were removed by centrifugation (15 min, 15000 rpm, JA-20, RT) and the clear supernatant loaded on 1 ml NiNTA-agarose column (preequilibrated in buffer A) at a flow rate of 1 ml/min using a peristaltic pump. Column was washed with 10 ml buffer B (100 mM NaH_2PO_4, 10 mM TRIS, 8 M urea, pH 8.0) followed by 10 ml buffer C (100 mM NaH_2PO_4, 10 mM TRIS, 8 M urea, pH 6.3). Bound proteins were eluted with 10 ml buffer D (100 mM NaH_2PO_4, 10 mM TRIS, 8 M urea, pH 4.5). Protein purification was monitored by SDS-PAGE (see 2.4.3.1) and CBB staining (see 2.4.3.2.). If the protein was to be coupled to the CNBr-activated Sepharose, all the buffers contained 6 M guanidium-HCl instead of 8 M urea due to the incompatibility of the latter with the coupling procedure.

b) Purification under native conditions

Proteins which were soluble in *E. coli* cytosol were normally purified under native conditions. The whole procedure was done at 4°C to prevent proteolysis. Bacterial cell pellet was resuspended in 25 ml buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), supplemented with 1 mM PMSF and 1 mg/ml lysozyme and stirred for ca. 45 min at 4°C. Cell walls and released DNA were completely broken by sonication as described above (see 2.4.1.2). Cell debrices were removed by centrifugation (15 min, 15000 rpm, JA-20, 4°C) and the clear supernatant loaded on an 1 ml NiNTA-agarose

column, preequilibrated with buffer A, at a flow rate of 1 ml/min using a peristaltic pump. Column was washed with 10 ml buffer A and bound proteins eluted with 10 ml buffer A with imidazole concentration increased to 300 mM. Purity of the recombinant protein was checked by SDS-PAGE (see 2.4.3.1) and CBB staining (see 2.4.3.2.).

2.4.2. Preparation of affinity matrices for antibody purifications

2.4.2.1. Coupling of peptides to the SulphoLink beads

Peptide antibodies against Tim proteins were purified on affinity columns with corresponding peptides coupled. An additional cysteine was inserted at the N terminus of all peptides to enable their coupling to the SulfoLink gel (Pierce).

One ml SulfoLink gel was packed into an appropriate column and the beads equilibrated with 6 ml 50 mM TRIS/HCl, 5 mM EDTA, pH 8.5. Column was closed at the bottom and 1 mg of peptide, dissolved in 1 ml of equilibration buffer, added. Column, closed at both ends, was gently rolled for 15 min at RT and then incubated for additional 30 min at RT without mixing. Buffer was drained and the beads washed with 3 ml equilibration buffer. Excess of reactive groups was blocked with 1 ml 50 mM cysteine. Column was mixed with the cysteine solution for 15 min at RT and then incubated for another 30 min at RT without mixing. All nonspecifically bound peptide was removed by a washing step with 16 ml 1 M NaCl. Column was stored at 4°C in 0.05% Na-azide.

2.4.2.2. Coupling of recombinant proteins to the CNBr-activated Sepharose

Antibodies to Tim proteins were purified on the affinity columns made by coupling the corresponding antigens to the CNBr-activated Sepharose 4B (Amersham). Proteins are covalently bound to the CNBr-activated Sepharose via their amino groups and it was therefore necessary to remove all other amino group-containing substances from protein solution prior to coupling. This was done by exchanging the buffer on the PD-10 column (Amersham). The column was first equilibrated with 30 ml 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (and 6 M guanidium-HCl, if the protein to be coupled was purified under denaturing conditions). Protein solution (containing ca. 5 mg protein) was adjusted, if needed, with equilibration buffer to 2.5 ml and loaded on the column by a gravity flow. These 2.5 ml were allowed to pass through and the protein was collected from the column in the next 3.5 ml. The sample was ready for coupling.

0.4 g of the CNBr-Sepharose (which will give ca. 1.5 ml gel) was allowed to swell in 5 ml 1 mM HCl for 45 min. Gel was washed with 200 ml 1 mM HCl on a sintered glass filter and transferred into an appropriate column. The final packed volume was ca. 1 ml. Remaining HCl solution was allowed to pass through and the column closed at the bottom. Protein solution was added, column closed on the top and gently tumbled at RT. After 1 h, column was put in the upright position, buffer allowed to pass through (can be kept to check the efficiency of coupling) and the beads washed with 6 ml 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (at this step proteins are presumed coupled so that guanidium-HCl is no longer necessary). Remaining active groups were blocked with 0.1 M ethanolamine, pH 8.0. Six ml were loaded on the column, 2 ml were allowed to pass through before the column was closed and gently tumbled at RT for 2 h. All nonspecifically and noncovalently bound proteins were removed by 3 washing cycles of alternating pH. Each cycle consisted of 6 ml each 0.1 M Na-acetate, 0.5 M NaCl, pH 4.5 and 0.1 M TRIS/HCl, 0.5 M NaCl, pH 8.0. Column was finally washed with 10 ml 10 mM TRIS/HCl, pH 7.5, 0.05% Na-azide and kept in this buffer at 4°C.

2.4.3. Analytical protein methods

2.4.3.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to their sizes on a vertical, discontinuous SDS-PAGE system (Laemmli, 1970). Two different electrophoresis systems were used. Normally, big gels were used (running gel: 9 x 15 x 0.1cm; stacking gel: 1 x 15 x 0.1 cm). Mini gels (Mini-PROTEAN II, Bio-Rad)(running gel: 7 x 7.2 x 0.075 cm; stacking gel: 1 x 7.2 x 0.075 cm) were run less frequently. Acrylamide concentration in the running gel was chosen according to the sizes of proteins to be separated.

Running gel	8-16% (w/v) acrylamide, 0.16-0.33% (w/v) bis-acrylamide, 375 mM TRIS/HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED
Stacking gel	5% (w/v) acrylamide , 0.1% (w/v) bis-acrylamide, 60 mM TRIS/HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED
Electrophoresis buffer	50 mM TRIS, 384 mM glycine, 0.1% (w/v) SDS pH 8.3 without adjustment

Protein samples were dissolved in 1 x Laemmli buffer (60 mM TRIS/HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromphenol-blue) and heated for 3 min at 95°C before loading on the gel. Big gels were run at 35 mA and mini gels at 25 mA until the blue front reached bottom of the gel (ca. 1.75 h for big and ca. 50 min for mini gels). Separated proteins were either stained with CBB (2.4.3.2.) or silver (2.4.3.3.) or transferred onto a nitrocellulose membrane (2.4.3.4.)

2.4.3.2. Staining SDS-PA gels with Coomassie Brilliant Blue (CBB)

SDS-PA gel to be stained with CBB was briefly washed with water and then immersed into the CBB staining solution (0.1% (w/v) CBB G- or R-250, 40% (v/v) methanol, 10% (v/v) acetic acid). Staining can be speeded up by heating the solution for 1 min in the microwave. Background staining was removed by repeated washes in 40% (v/v) methanol, 10% (v/v) acetic acid.

2.4.3.3. Silver staining of SDS-PA gels

(Blum *et al*., 1987)

SDS-PA gel was briefly washed with water and the proteins fixed for 30-60 min in the fixing solution (50% methanol, 10% acetic acid and 50 µl freshly added 37% formaldehyde pro 100 ml solution). Gel was washed with 50% ethanol for 2 x 10 min and then immersed for 1 min in 0.02% (w/v) $Na_2S_2O_3$. After 3 brief (20 s each) washing steps with water, the gel was incubated for 15 min in a freshly prepared 0.2% (w/v) $AgNO_3$ with 75 µl 37% formaldehyde added pro 100 ml. Excess of silver was removed by 2 short washing steps with water and the gel "developed" in 6% (w/v) Na_2CO_3 solution with freshly added 2 ml 0.02% (w/v) $Na_2S_2O_3$ and 50 µl 37% formaldehyde pro 100 ml solution. Reaction was stopped by briefly washing with water and incubating the gel in 50 mM EDTA, pH 8.0.

2.4.3.4. Transfer of proteins onto nitrocellulose membranes and Ponceau S staining

Proteins separated on SDS-PA gel were electrophoretically transferred on a nitrocellulose membrane according to (Towbin *et al*., 1979) and (Khyse-Anderson, 1984). A gel, a nitrocellulose membrane and Whatman 3MM papers were immersed in the blotting buffer

(20 mM TRIS, 150 mM glycin, 20% (v/v) methanol, 0.08% SDS). A sandwich, consisting of 3 Whatman 3MM papers, a nitrocellulose membrane (0.2 μm pore size), a PA gel and another 3 Whatman 3MM papers, was assembled between two graphite electrodes and proteins were transferred onto the nitrocellulose membrane at 250 mA for 75 min (for big gels) or at 110 mA for 40 min (for mini gels). Transferred proteins were visualized by Ponceau S (0.2% (w/v) Ponceau S in 3% (w/v) TCA).

2.4.3.5. Protein precipitation with trichloracetic acid (TCA)

Proteins were precipitated out of solution by addition of 12% (w/v) TCA. Samples were kept for 30 min at -20°C and centrifuged for 20 min at 20000 rpm (Sigma centrifuge). Pellet was washed with ice-cold acetone and, after brief drying at RT, resuspended in 1 x Laemmli buffer.

2.4.3.6. Determination of protein concentration

Protein concentration was determined according to (Bradford, 1976) using the "Bio-Rad-Proteinassay" reagent. Protein solution was mixed with 1 ml 1:5 diluted reagent and incubated for 10 min at RT. Absorbance at 595 nm was read and protein concentration determined from a calibration curve made with the known amounts of bovine IgGs as a standard.

2.4.3.7. Detection and quantification of radiolabeled proteins

Radiolabeled proteins were detected by autoradiography. A dried gel or a nitrocellulose membrane was exposed to an X-ray film (Kodak Bio Max MM) and, after a desired period of exposure, film was developed in a developing machine (Gevamatic 60, AGFA-Gevaert). Period of exposure depended on signal intensities. Signals were quantified with Image Master 1D Elite software (Amersham).

2.5. Methods in cell biology

2.5.1. Methods in *N. crassa* cell biology

2.5.1.1. "Fast mito preps"

Expression of His-tagged proteins in various *N. crassa* transformants was quickly checked by "fast mito preps". 50 ml *N. crassa* cultures were grown overnight at 25°C under constant agitation. Hyphae were collected by vacuum filtration and transferred to 2 ml Eppendorf cups already containing 1.3 ml SEM buffer (0.25 M sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) and 200 µl sea-sand. Cells were disrupted in Ribolyzer Cell Disruptor (Hybaid AGS) for 2 x 45 s at the maximum power. Non-broken cells, cell debrices and sand were removed by 2 centrifugation steps for 5 min at 5000 rpm (Sigma centrifuge). Mitochondria were pelleted by centrifugation for 15 min 15000 rpm (Sigma centrifuge) and mitochondrial pellet resuspended in 50 µl SEM buffer. Usually 15 µl of this suspension was loaded on an SDS-PA gel for analysis.

2.5.1.2. Isolation of mitochondria

Mitochondria were usually isolated from a 2 l overnight culture. Hyphae were collected by vacuum filtration and their wet weight determined (usually about 20 g). After chopping into smaller pieces, hyphae were mixed with 20 ml of sea-sand and 20 ml of SEMP (SEM buffer with 1 mM PMSF added) in a mortar. Hyphae were grinded with a pestle for ca. 1 min. Additional 20 ml of SEMP was added and the mixture grinded to homogeneity (ca. 1 min). The third 20 ml of SEMP was added to make the mixture more liquid. After a brief additional grinding, homogenate was transferred to JA-20 tubes and non-broken cells, sand and nuclei pelleted by 5 min centrifugation at 5000 rpm in JA-20 rotor (Beckman). This centrifugation step was repeated once again and mitochondria then pelleted from the supernatant (12 min, 12000 rpm, JA-20). Mitochondrial pellet was resuspended in 1 ml SEMP, diluted with ca. 20 ml SEMP and pelleted once again (12 min, 12000 rpm, JA-20). Final mitochondrial pellet was resuspended in 1 ml SEM, protein concentration determined by Bradford assay and mitochondria, if necessary, diluted to 10 mg/ml with SEM. Mitochondria were either immediately used or frozen in liquid nitrogen and kept at -70°C. Usual yield of mitochondria from a 2 l culture is ca. 25 mg.

2.5.1.3. "Big preps"

When large amounts of mitochondria were needed, *N. crassa* was grown in 100 l containers yielding between 1000 and 2000 g hyphae. They were not grinded by hand but in a cell-mill (Sebald *et al*., 1979). Hyphae were chopped by hand and mixed with 3 l SEMP and 2000 g sea-sand in a Waring blendor (3x 10 s). Obtained paste was passed twice through a cell-mill and mitochondria isolated by differential centrifugation as described above but using rotors with bigger capacities.

HisTim23 and OxaHis strains are growing badly under these semi-sterile conditions. They were grown as five 8 l-cultures yielding about 700 g hyphae in total. Mitochondria were afterwards isolated as in usual "big preps".

2.5.1.4. Digitonin fractionation

(Segui-Real *et al*., 1993).

Freshly isolated *N. crassa* mitochondria were resuspended at 5 mg/ml in ice-cold SEMK buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, 80 mM KCl, pH 7.2) supplemented with different concentrations of digitonin (0-0.5%) and incubated with or without proteinase K (50 µg/ml) for 5 min on ice. As a control one sample was completely solubilized with Triton X-100. Lysis of mitochondria was stopped by diluting samples 5-fold with ice-cold SEMK buffer. Proteolysis was continued for another 30 min on ice and then stopped by addition of 1mM PMSF. Samples were precipitated with 12% trichloracetic acid and analyzed by SDS PAGE and immunodecoration.

2.5.2. Methods in yeast cell biology

2.5.2.1. Isolation of yeast mitochondria

Yeast cells, grown until OD_{600} 1-1.2, were collected by centrifugation (5 min, 4000 rpm, JA-10), washed once with water and, after determination of their wet weight, resuspended in 100 mM TRIS/SO_4, pH 9.4, 10 mM DTT at 0.5 g/ml. After 15 min shaking at 30°C, cells were collected as described above, washed once with ca. 200 ml 1.2 M sorbitol and resuspended in zymolyase buffer (1.2 M sorbitol, 20 mM K-phosphat, pH 7.4, 4 mg zymolyase per g cells) at 0.15 g/ml. This suspension was shaken for 30-60 min at 30°C. Efficiency of lysis was checked after 30 min by diluting 25 µl of suspension in either 1 ml

water or 1 ml 1.2 M sorbitol. Lysis was stopped if the OD_{600} of the water suspension was 10-20% of the sorbitol one.

All following steps were done on ice and as fast as possible. Spheroplasts were collected by centrifugation (5 min, 35000 rpm, JA-10) and resuspended at 0.15 g/ml in homogenization buffer (0.6 M sorbitol, 10 mM TRIS/HCl, pH 7.4, 1 mM EDTA, 0.2% (w/v) fatty acid-free BSA, 1 mM PMSF). Spheroplasts were opened by 10 strokes in a Dounce-homogenizator. Cell debrices, nonbroken cells and nuclei were removed by centrifugation (2 x 5 min, 4000 rpm, JA-20). Mitochondria were pelleted from the supernatant (12 min, 12000 rpm, JA-20) and resuspended in 25 ml SH buffer (0.6 M sorbitol, 20 mM HEPES/KOH, pH 7.3). After a centrifugation step (5 min, 4000 rpm, JA-20), mitochondria were isolated from the supernatant by centrifugation (12 min, 12000 rpm). Final mitochondrial pellet was resuspended in 1 ml SH buffer and the protein concentration determined by Bradford assay. Mitochondria were diluted to 10 mg/ml, aliquoted into 50 µl aliquots, frozen in liquid nitrogen and stored at -70°C.

2.5.2.2. Preparation of mitoplasts

Mitochondria whose outer membrane has been disrupted are known as mitoplasts. The outer mitochondrial membrane is specifically opened by swelling of mitochondria in a hypotonic solution which keeps the inner membrane intact. Mitochondria in SH buffer were diluted 1:10 in 20 mM HEPES/KOH, pH 7.3 containing 1 mM ATP and incubated on ice for 20 min. As a control, mitochondria were diluted in SH buffer in the same manner. If necessary, mitoplasts (or mitochondria) were reisolated by centrifugation (10 min, 14000 rpm) and resuspended in a desired buffer.

2.5.2.3. In vitro import into mitochondria

Precursor proteins were synthesized in the presence of ^{35}S-methionine in the standard or in the coupled reticulocyte lysate system (Promega). Mitochondria were resuspended at 0.5 mg/ml in import buffer (0.05% (w/v) fatty acid- free BSA (w/v), 600 mM sorbitol, 50 mM HEPES/KOH, 80 mM KCl, 10 mM $MgAc_2$, 2.5 mM EDTA, 2 mM K-phosphate and 1 mM $MnCl_2$, pH 7.2) with further addition of 2 mM NADH, 1 mM ATP, 10 mM creatine phosphate and 100 µg/ml creatine kinase. Import was started by addition of lysate (1-3% (v/v)) and continued for different time points at 25°C (or in rare cases at 12°C). Samples

were divided and diluted either in SH-buffer (600 mM sorbitol, 20 mM HEPES-KOH, pH 7.2) or SH-buffer plus 50 µg/ml proteinase K and incubated 15 min on ice. Protease treatment was stopped by addition of 2 mM PMSF. Mitochondria were reisolated and the import reaction analyzed by SDS-PAGE, transfer to nitrocellulose membrane, autoradiography and quantification.

2.5.2.4. Generation of translocation intermediates

^{35}S-labeled precursor protein, $pb_2\Delta19(167)DHFR_{K5}$ was arrested in wild type yeast mitochondria in three different import stages - at the level of the outer membrane, as an intermediate spanning both mitochondrial membranes and fully imported into the matrix.

To arrest the precursor at the level of the outer membrane, mitochondria (0.5 mg/ml) were incubated with 6 µM oligomycin, 25 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 0.5 µM valinomycin for 10 min at 25°C to dissipate membrane potential before precursor protein was added. To arrest the precursor protein as an intermediate spanning both membranes, precursor protein was preincubated with methotrexate (MTX) (1 µM) and NADPH (3 mM) and then imported into energized mitochondria in the presence of 2 µM MTX and 5 mM NADPH. Precursor was completely imported into the matrix as described under 2.5.2.3. Following the import reactions, samples were either directly analyzed by SDS-PAGE and/or subjected to crosslinking and immunoprecipitations.

Same procedure was used to generate a two-membrane spanning intermediate in *N. crassa* mitochondria.

In the chase experiments, precursor was first arrested at the level of the outer membrane and afterwards chased into the matrix. Mitochondria were deenergized by addition of 50 µM CCCP for 10 min at 25°C before $pb_2\Delta19(167)DHFR_{K5}$ was added. Mitochondria were reisolated to remove all nonbound precursors and membrane potential then reestablished by addition of 2 mM DTT and 5 mM NADH. After different time periods, aliquots were removed and either directly analyzed for fully imported protein or subjected to cross-linking and immunoprecipitation.

2.5.3. Protease treatments

After the import reaction, nonimported precursors as well as those only partially translocated across the outer membrane can be degraded by externally added protease. Furthermore, protease digestion can be done in mitochondria or in mitoplasts thus enabling submitochondrial localization of the imported protein.

Import mix was split on ice into 3 aliquots. One was diluted 1:10 into SH buffer, the second one into SH buffer containing 50 µg/ml proteinase K and the third one into 20 mM HEPES/KOH, pH 7.3 containing 50 µg/ml proteinase K. Samples were kept on ice for 15 min after which protease digestion was stopped by 1 mM PMSF. Mitochondria were reisolated, resuspended in Laemmli buffer and samples analyzed by SDS-PAGE and autoradiography. Protease accessibility of the imported protein was compared to marker proteins of the outer and inner membranes and of the matrix.

The same procedure can be done directly with mitochondria thus enabling the localization of endogeneous proteins.

2.5.4. Crosslinking

2.5.4.1. Crosslinking of mitochondrial proteins

During this work, following crosslinkers were used: DFDNB (1,5-difluoro-2,4-dinitrobenzene), DSG (disuccinimidylglutarate) and DSS (disuccinimidylsuberate). They are all membrane permeable, lysine-specific crosslinkers with different spacer arms (0.3-1.14 nm).

Mitochondria were resuspended in the import buffer (without BSA) and a crosslinker was added from a 100-fold stock in DMSO. After 30 min on ice, excess crosslinker was quenched with 100 mM glycine, pH 8.8. Mitochondria were reisolated and analyzed by SDS-PAGE and immunodecoration. Where indicated, mitochondrial ATP was depleted prior to crosslinking by addition of apyrase (1 U/ml) and oligomycin (10 µM) or kept high by addition of ATP (4 mM), NADH (5 mM), creatine-phosphate (10 mM) and creatine-kinase (0.1 mg/ml) for 10 min at 25°C. When crosslinking adducts were analyzed for binding to the NiNTA-agarose beads, reisolated mitochondria were solubilized with 1% SDS in 50 mM Na-phosphate, pH 8.0, 100 mM NaCl, 10% glycerol, 10 mM imidazole, 1 mM PMSF for 15 min at 25°C. Samples were diluted 20 fold in the same buffer containing 0.2% Triton X-100 and, after a clarifying spin, added to 50 µl NiNTA-agarose

beads. After 1 h agitation at 4°C, beads were washed and bound proteins eluted with Laemmli buffer containing 300 mM imidazole for 5 min at 95°C.

2.5.4.2. Crosslinking of translocation intermediates

When precursor proteins, arrested at different import stages, are subjected to crosslinking, they become covalently bound to the neighboring proteins. The changing environment of the precursor during translocation can thus be followed and the neighboring proteins identified by immunoprecipitations.

Precursor proteins were arrested at 3 different import stages as described under 2.5.2.4. Samples were cooled on ice and a crosslinker added from a 100 fold stock in DMSO. After 30 min, crosslinker was quenched by addition of 100 mM glycine, pH 8.8. Mitochondria were reisolated, solubilized in SDS-containing buffer and crosslinked products were analyzed by immunoprecipitation, SDS-PAGE and autoradiography.

2.6. Methods in immunology

2.6.1. Overview of antibodies made

Antibody	Antigen	Rabbit
αTim17 (N.c)	C-term. peptide (C-EAPAPPPSNEKVLA)	135
αTim23N (N.c)	MBP-Tim23(1-106)	204
αTim23C (N.c)	C-term. peptide (C-RRTFFPSPQTNEVD)	217
αTim50 (N.c)	His_6-Tim50(189-540)	291, 292
αTim14 (S.c)	His_6-Tim14(50-168)	313, 315

2.6.2. Generation of polyclonal antisera in rabbits

Polyclonal antisera were made in rabbits. Recombinant proteins expressed in bacteria or synthetic peptides coupled to the carrier protein (KLH) were used as antigens. Purified recombinant proteins were loaded on SDS-PA gels, blotted on nitrocellulose membranes and stained with Ponceau S. Pro injection, 5-10 bands (10-100 µg protein) were cut and the nitrocellulose dissolved in 300 µl DMSO (Knudsen KA, 1985). Dissolved proteins

were mixed with 300 µl Freunds incomplete adjuvant (or Injection TiterMax™ for the first injection) until a stable emulsion was obtained. This emulsion was subcutaneously injected into a rabbit. The antigen was injected twice within ten days before the first bleeding was taken.

Ten days after each injection, 30 - 40 ml blood was taken from the ear vane and left at RT for ca. 2 h. After coagulation was complete, serum was obtained by two centrifugation steps (15 min, 4000 rpm, JA-10 and then 10 min, 12000 rpm, JA-20 rotor). Complement was inactivated by a 20 min incubation at 56°C and the serum stored at -20°C.

2.6.3. Affinity purification of antibodies

Specific antibodies were affinity purified from the sera using antigen-affinity columns (see 2.4.2.1. and 2.4.2.2.). Six ml serum was diluted with 24 ml 10 mM TRIS/HCl, pH 7.5 and loaded on the corresponding affinity column under gravity flow at RT. Column was washed with 10 ml 10 mM TRIS/HCl, pH 7.5 followed by 10 ml 10 mM TRIS/HCl, 0.5 M NaCl, pH 7.5. Specific antibodies were eluted with 10 ml 0.1 M glycine/HCl, pH 2.5. Fractions were immediately neutralized with 1M TRIS/HCl, pH 8.8, aliquoted and stored at -20°C.

2.6.4. Immunodetection of proteins on nitrocellulose membranes

Proteins transferred onto nitrocellulose membranes were identified by immunodecoration with specific antibodies. Membranes were first incubated for 1 h in 5% (w/v) milk powder in TBS (135 mM NaCl, 10 mM TRIS/HCl, pH 7.5) to block all nonspecific binding sites. Blocking solution was removed and a primary antibody added. Depending on the titer, primary antibodies were diluted 1:200 to 1:10000 in 5% milk in TBS and incubated from 1 h to overnight. Membranes were then washed for 10 min in TBS, 10 min in TBS/0.05% Triton X-100 and again 10 min in TBS. Bound antibodies were detected with goat anti-rabbit antibodies coupled to horseradish peroxidase. This secondary antibody was diluted 1:10000 in 5% milk in TBS and incubated for 1 h. Blots were washed as described above, the chemiluminescent substrate of peroxidase added and the signals detected on X-ray films.

For detection of His-tagged proteins, Penta-His antibody (Qiagen) was used. Membranes were blocked in 3% (w/v) fatty acid-free BSA in TBS and Penta-His antibody diluted

1:1000 in this blocking solution. Secondary antibody (goat anti-mouse) was diluted 1:5000 in 5% (w/v) milk powder in TBS. Blots were developed as described above.
For detection of HA-tagged Tim14, anti-HA antibody (Roche) was used. Blots were blocked in the supplied blocking solution. Secondary antibody (goat anti-mouse) was diluted 1:5000 in 5% (w/v) milk powder in TBS. Blots were developed as described above.

Solution 1	3 ml TRIS/HCl, pH 8.5 (1M stock), 300 µl luminol (440 mg/10 ml DMSO), 133 µl p-coumaric acid (150 mg/10 ml DMSO), H_2O to 30 ml
Solution 2	3 ml TRIS/HCl, pH 8.5 (1M stock), 18 µl H_2O_2 (30%), H_2O to 30 ml

Solutions are stable for ca. 1 week if kept in light-protected bottles at 4°C. Chemiluminescent substrate was made by mixing equal volumes of solution 1 and solution 2.

2.6.5. Immunoprecipitations under stringent conditions

Crosslinking adducts of translocation intermediates were identified by immunoprecipitations under stringent conditions with antibodies against translocation components. Antibodies were bound to TBS-washed Protein A-Sepharose CL-4B (PAS) by a 2 hour overhead incubation at 4°C. Fifty µl beads and enough antibodies to imunodeplete corresponding antigen from the extract were used pro immunoprecipitation. Whenever possible, affinity purified antibodies were used. If the whole serum was used, antibodies were crosslinked to PAS beads before use in immunoprecipitations (see 2.6.7).
Mitochondrial pellet was solubilized at 5-10 mg/ml with 1% SDS in 50 mM Na-phosphate, pH 8.0, 100 mM NaCl, 1 mM PMSF for 15 min at RT. Samples were diluted 20 fold in the same buffer containing 0.2% Triton X-100 and the nonsolubilized material removed by an ultracentrifugation step. Supernatants were added to the antibodies prebound to PAS beads and the mixture incubated overhead for 2 h at 4°C. Beads were washed twice with TBS/0.2% Triton X-100 and once with TBS before bound material was eluted with nonreducing Laemmli buffer (5 min at 95°C). Samples were analyzed by SDS-PAGE and autoradiography.

2.6.6. Coimmunoprecipitations

If the composition of protein complexes was analyzed by immunoprecipitations, mitochondria (2 mg/ml) were solubilized with 1% (w/v) digitonin in 50 mM Na-phosphate, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF for 30 min at 4°C. After a clarifying spin, mitochondrial extract was added to antibodies prebound to PAS beads and further processed as described under 2.6.5. except that the washing steps were done with the solubilization buffer containing 0.05% (w/v) digitonin. In some cases, 20 mM TRIS/HCl, pH 7.4, 80 mM KCl, 5 mM EDTA was used for solubilization of yeast mitochondria and for washing steps afterwards. Samples were in the end analyzed by SDS-PAGE and immunodecoration.

2.6.7. Covalent binding of antibodies to Protein A-Sepharose CL-4B

When affinity purified antibodies were not available, antibodies from the whole serum were bound to PAS beads as described under 2.6.5. To prevent their subsequent elution from the beads, they were covalently bound to Protein A by crosslinking. After a binding step, PAS beads were washed 3 times with 0.1 M Na-borate, pH 9.0 and resuspended in 1.3 ml of the same buffer. Twelve mg of dimethylpimelimidate was dissolved in 100 µl borate buffer and added to the PAS suspension. After 30 min shaking at RT, beads were washed with 1 ml 0.2 M ethanolamine, pH 8.0, resuspended in 1 ml same ethanolamine solution and shaken for 2 h at RT to quench the excess of crosslinker. Beads were finally washed 3 times with TBS and stored at 4°C until use.

3. RESULTS

3.1. TIM23 complex of *Neurospora crassa*

3.1.1. Identification of the *tim23* gene in *N. crassa*

The TIM23 complex is responsible for the translocation of the vast majority of mitochondrial proteins across or into the mitochondrial inner membrane. In yeast, its known components are the integral membrane proteins Tim17 and Tim23, and membrane associated Tim44. Is this the complete set of components? To check this, I have set out to purify the TIM23 complex from *N. crassa* mitochondria via a His-tag on one of its components. This strategy had proven powerful in the case of the TOM complex (Kunkele *et al.*, 1998), however it requires the knowledge of at least one of the components. At the beginning of this work only the *tim17* gene was known, but its His-tagged versions were not functional (F. Nargang, unpublished results). To identify the *N. crassa tim23* gene, the yeast Tim23 protein sequence was used as a query to search the available databases for homologues. A number of Tim23 proteins, including some from fungal species, were identified. *N. crassa* Tim23 was not present in the available sequences. As the sequence homology among fungal Tim23 proteins and higher eukaryotes was considerably low, only the former ones were used for the alignment (Figure 5). Based on the conserved regions identified, degenerate primers were developed and used to amplify gene fragments from *N. crassa* genomic DNA or its cDNA library by PCR. After one round of nested PCR, products of the expected sizes were cloned and sequenced, and shown to contain a fragment of the *tim23* gene. This fragment was used to generate a probe to screen a pMOcosX #X cosmid library (Orbach, 1994). A full length genomic sequence was obtained by sequencing the identified cosmid. An intron-less version of the *tim23* was amplified from the cDNA library using primers designed from the genomic sequence. This enabled unambiguous identification of the predicted intron. Figure 6 shows the nucleotide sequence of the gene with the its flanking regions as well as the deduced amino acid sequence of *N. crassa* Tim23.

Similar approaches were used to identify *tim17* and *tim44* genes (F. Nargang, unpublished results and C. Kozany, unpublished results).

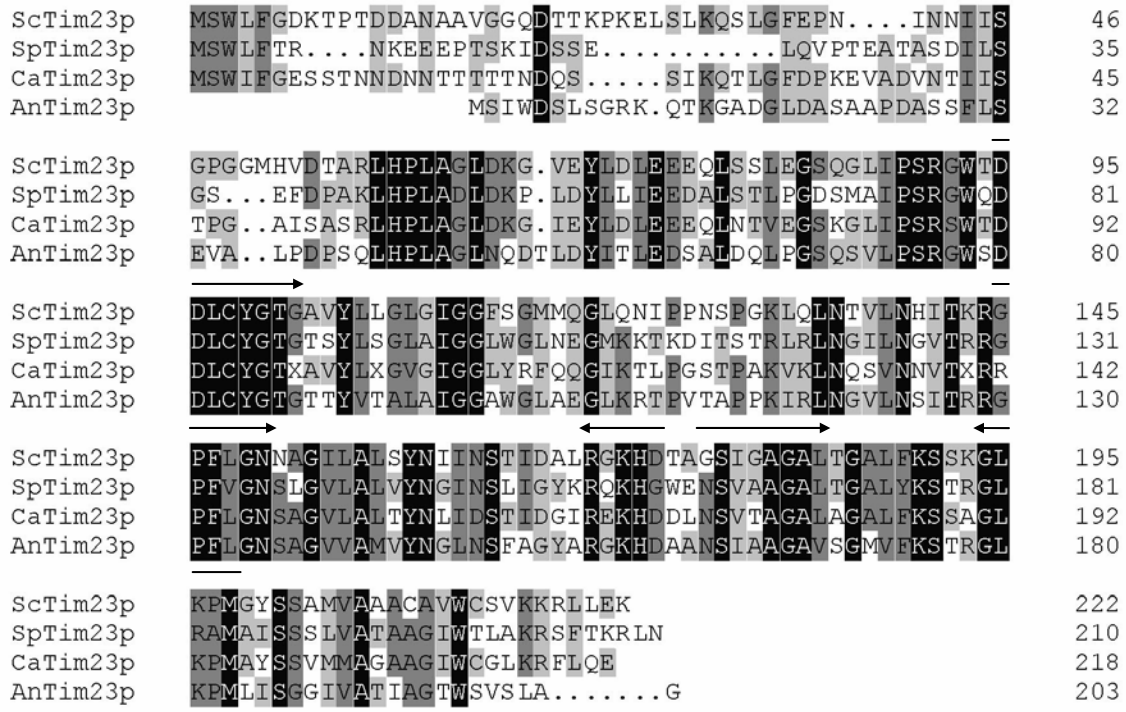

Figure 5. Alignment of the fungal Tim23 proteins

Tim23 protein of *S. cerevisiae* was used as a query to search the available databases for homologues. The alignment shows the identified Tim23 proteins from different fungal species. Sc *Saccharomyces cerevisiae*; Sp *Schizosaccharomyces pombe*; Ca *Candida albicans*; An *Aspergillus nidulans*; Arrows indicate regions used to design degenerate primers and their orientation.

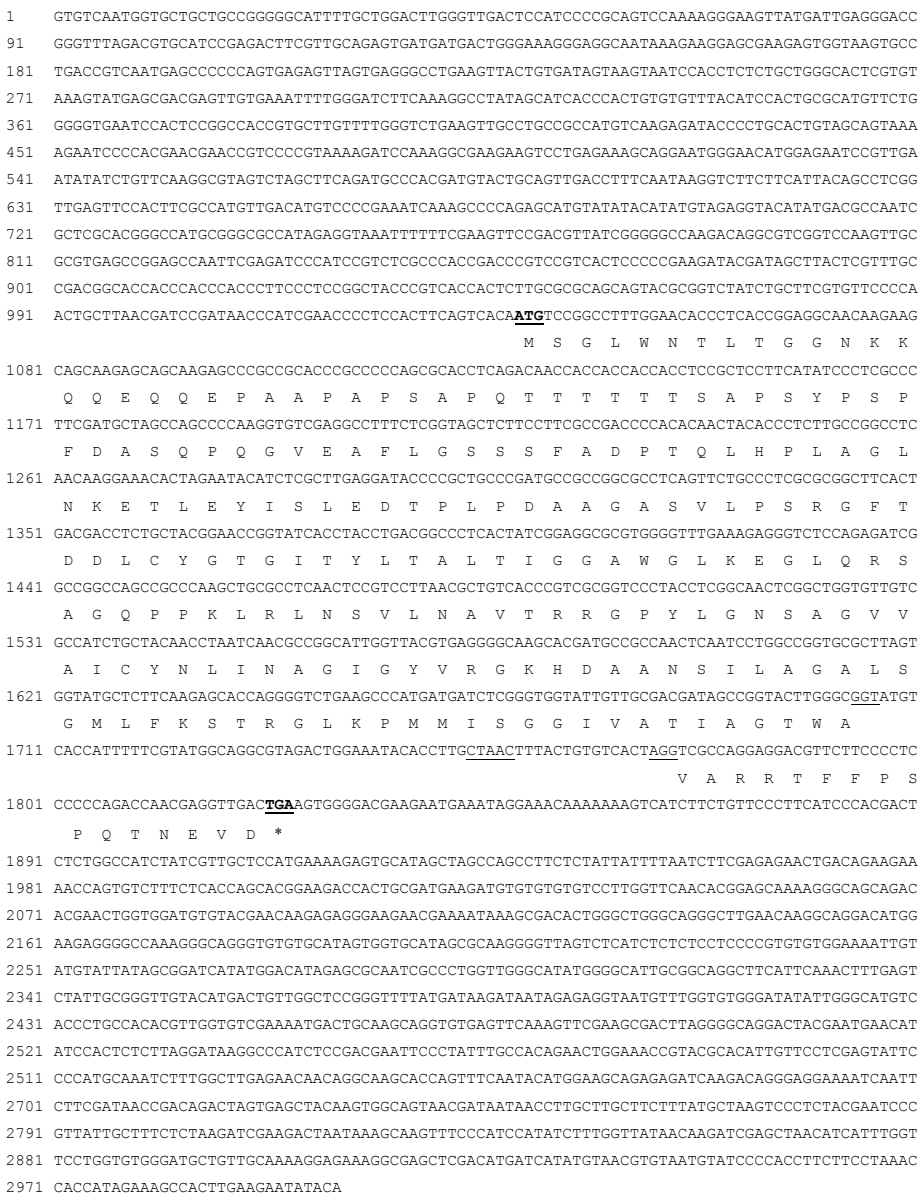

Figure 6. The nucleotide sequence of *N. crassa tim23* and the deduced amino acid sequence

A cosmid containing *tim23* gene was sequenced starting from the regions identified in the PCR screen with the degenerated primers. The position of the intron was identified by comparison of the genomic sequence with the sequence obtained from the cDNA library. Start and Stop codons are underlined and shown in bold. Specific intron flanking sequences are underlined.

3.1.2. Localization of Tim17, Tim23 and Tim44 in mitochondria

To determine the subcellular location of the newly identified *N. crassa* Tim proteins, total cell extracts were made, fractionated by differential centrifugation and obtained fractions analyzed by SDS-PAGE and immunoblotting. For immunodetection, antibodies against Tim proteins were raised in rabbits and affinity purified before use. All Tim proteins were exclusively found in the mitochondrial fraction (Figure 7A). When mitochondria were subjected to carbonate extraction, which enables separation of integral from peripheral membrane proteins and soluble ones, Tim17 and Tim23 behaved like the ATP/ADP carrier (AAC) showing that they are integral membrane proteins. Tim44 was completely carbonate-extractable indicating that it is not an integral membrane protein (Figure 3B). However, when mitochondria were sonicated in the presence of increasing amounts of salt, about half of total Tim44 always co-fractionated with mitochondrial membranes, even in the presence of 1.5 M NaCl (Figure 7B). The other half of Tim44 molecules was always found soluble in the matrix. Apparently, Tim44 exists in two populations in mitochondria, one stably attached to the membrane and a soluble one.

To further sublocalize Tims within mitochondria and to determine their topologies, mitochondria were subjected to digitonin fractionation (Segui-Real *et al.*, 1993). In this procedure, mitochondria are incubated, in the presence of proteinase K, with increasing concentrations of digitonin to sequentially open outer and inner membrane so that protease can get access to specific mitochondrial subcompartments. None of the Tims was accessible to the protease in intact mitochondria (Figure 7C). Tim17 and Tim23 were degraded at the same digitonin concentration as was cytochrome c heme lyase (CCHL) indicating that they are exposing domains into the intermembrane space (IMS). Antibodies directed against either N or C terminus of Tim23 revealed no fragments suggesting that both termini of Tim23 are facing the IMS. Tim44 was degraded only after opening the inner membrane as it behaved like mitochondrial processing peptidase (MPP).

Taken together, these data indicate that both Tim17 and Tim23 are integral membrane proteins of the mitochondrial inner membrane. Both N and C termini of Tim23 are facing the IMS in agreement with the predicted four transmembrane topology. Considering the fact that Tim17 is also predicted to have four transmembrane domains and that its C terminus is facing the IMS it is reasonable to assume that its N terminus is located in the IMS as well. Tim44, on the other hand, is present as a soluble and a peripheral membrane protein in the mitochondrial matrix.

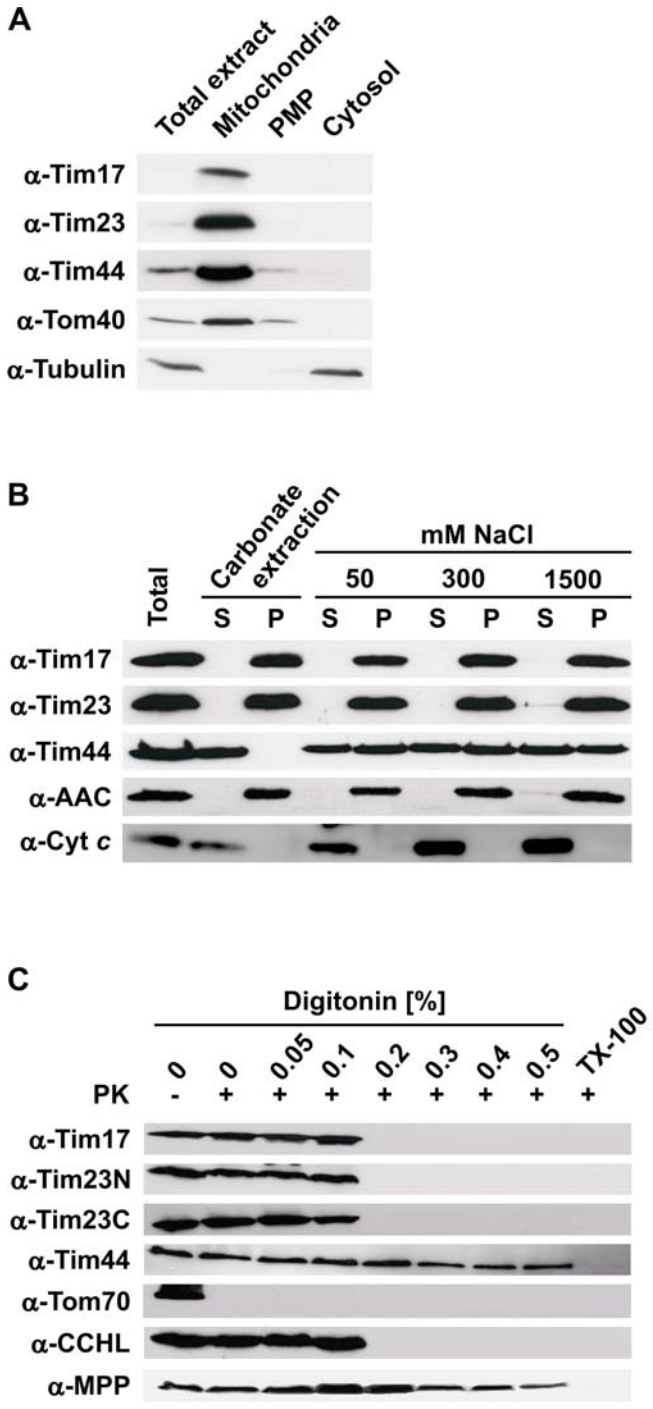

Figure 7. Subcellular localization of *N. crassa* TIM23 components

(**A**) Total cell extract was subjected to differential centrifugation and fractions corresponding to mitochondria, postmitochondrial pellet (PMP) and cytosol were analyzed by SDS-PAGE and immunoblotting. Antibodies against Tom40 and tubulin were used as markers for mitochondria and cytosol, respectively. (**B**) Membrane association of the Tim proteins was analyzed by carbonate and salt extractions. Samples corresponding to supernatants (S) and pellets (P) were analyzed by SDS-PAGE and immunoblotting. ATP/ADP carrier (AAC) and cytochrome *c* (Cyt *c*) were used as markers for integral membrane and soluble proteins, respectively. (**C**) Submitochondrial location of the Tim proteins was analyzed by digitonin fractionation. Mitochondria were treated, in the presence of proteinase K, with increasing concentrations of digitonin to successively open outer and inner membrane so that protease had access to different subcompartments. Samples were subjected to SDS-PAGE and analyzed by immunodecoration using Tom70, cytochrome *c* heme lyase (CCHL) and mitochondrial processing peptidase (MPP) antibodies as markers for the outer membrane, intermembrane space and the matrix, respectively. Mitochondria completely lysed with Triton-X-100 were used as a control.

3.1.3. *N. crassa* Tims are in close proximity to the translocating polypeptide

To determine if the *N. crassa* Tim proteins in are in close contact to the translocating precursor, a preprotein was arrested as an intermediate spanning both TOM and TIM23 complexes and its neighborhood analyzed by chemical crosslinking followed by immunoprecipitations. As a precursor, pcytb_2Δ19(167)DHFR$_{K5}$ was used (Schneider *et al.*, 1994). It consists of the first 167 residues of the precytochrome b_2, with the intact matrix targeting signal but deleted sorting signal, fused to the mouse DHFR (dihydrofolate reductase). Five lysine residues were added at positions 113-118 to increase the efficiency of crosslinking. When this precursor is imported into mitochondria in the presence of membrane potential ΔΨ, ATP and methotrexate (MTX), its N terminus is in the matrix while the C terminus is still outside of mitochondria due to the stably folded DHFR moiety. After generation of such intermediates, samples were subjected to crosslinking with 1,5-difluoro-2,4-dinitrobenzene (DFDNB) followed by immunoprecipitations with affinity purified antibodies to Tim17, Tim23 and Tim44 and a preimmune serum as a control. Figure 8 shows that all three proteins are indeed found in the neighborhood of the translocating chain as specific crosslinking adducts of the radiolabeled precursor could be immunoprecipitated with each of the Tim antibodies.

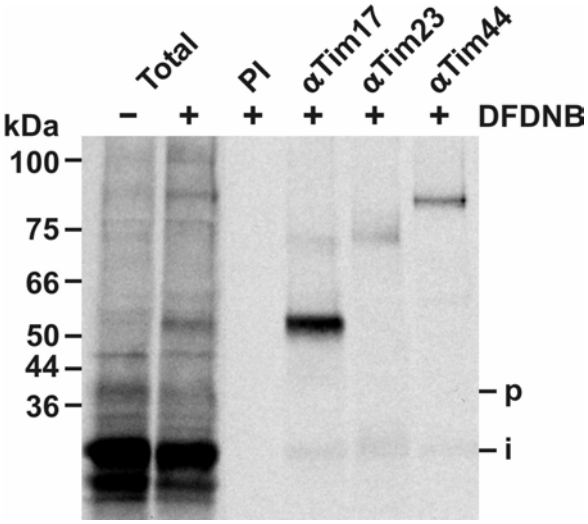

Figure 8. Crosslinking of the Tim proteins to the translocation intermediate

The precursor pcytb_2Δ19(167)DHFR$_{K5}$ was labeled with ^{35}S-methionine and imported into energized *N. crassa* mitochondria in the presence of methotrexate to arrest it as an intermediate spanning both mitochondrial membranes. Samples were subjected to crosslinking with DFDNB and, after solubilization under stringent conditions, immunoprecipitation was performed with affinity purified antibodies to Tim17, Tim23, and Tim44. Total crosslinking reaction and immunoprecipitates were analyzed by SDS-PAGE and autoradiography. p- precursor and i- intermediate forms of pcytb_2Δ19(167)DHFR$_{K5}$.

3.1.4. Coimmunoprecipitations of the TIM23 components

All Tim proteins apparently interact with a preprotein in transit but are they parts of the same complex? To test this, mitochondria were solubilized with digitonin and subjected to coimmunoprecipitation with affinity purified antibodies to Tim17, Tim23 and Tim44 (Figure 9). Tim17 antibodies immunodepleted both Tim17 and Tim23 from detergent extracts but no Tim44 was found in the immunoprecipitate. Essentially the same result was obtained with Tim23 antibodies. Antibodies to Tim44 efficiently immunoprecipitated Tim44, however neither Tim17 nor Tim23 was found in the immunoprecipitate. The same result was obtained when the solubilization buffer contained low (50mM) or high salt concentration (500mM). In conclusion, in *N. crassa* Tim17 and Tim23 form a subcomplex. Apparently, Tim44 is not stably associated with this subcomplex, at least not under the experimental conditions used.

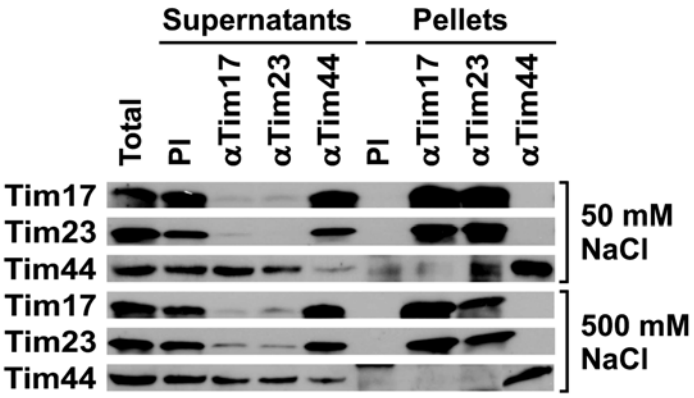

Figure 9.

Coimmunoprecipitation of *N. crassa* TIM23 components

Mitochondria were solubilized with 1% digitonin in the presence of either 50 or 500 mM NaCl. Detergent extracts were subjected to immunoprecipitation with affinity purified antibodies to Tim17, Tim23, Tim44, or preimmune serum prebound to protein A-sepharose. After washing steps, bound material was eluted with Laemmli buffer. Samples were analyzed by SDS-PAGE and immunoblotting

3.1.5. *N. crassa* strains expressing His-tagged Tim23

To enable purification of the TIM23 complex, *N. crassa* strains expressing His-tagged Tim23 were made. Constructs with either N- or C- terminally tagged Tim23 under the endogenous promoter were cloned and transformed into wild type *N. crassa* strain. These transformants were used to test the functionality of the tagged versions, as judged by coisolation of Tim17 on the NiNTA-agarose beads, and for comparison of the binding efficiencies of N- and C-terminal tags. Both tagged versions enabled co-isolation of Tim17, however the N-terminal tag was binding significantly more efficiently to the NiNTA-agarose beads, probably due to the better accessibility of the tag (not shown). It was, therefore, chosen for further experiments. Interestingly, it was never possible to co-isolate the wild type version of Tim23 which was still present in these mitochondria, suggesting either a very labile interaction between two Tim23 molecules or that the complex indeed contains only one molecule of Tim23.

To unambiguously show the functionality of the tagged Tim23 and generate a strain expressing only the tagged version, a construct containing the N-terminally His-tagged Tim23 was transformed into *tim23* null mutant. This mutant was made in collaboration with Professor F. Nargang (University of Alberta, Canada) using the sheltered RIP procedure (Harkness *et al.*, 1994; Selker, 1990). As *tim23* gene is essential for cell viability, the null allele is present in a heterokaryon in which the normal copy of the gene, present in another nucleus, shelters the cell against the lethal effect of the mutation. In the case of *tim23* RIP strain, the two nuclei in the heterokaryon can be differentiated by their auxotrophies for tryptophan and adenine (Figure 10A). Non-functional *tim23* allele was present in the tryptophan-requiring nucleus. After transformation of this heterokaryotic strain with the plasmid encoding N-terminally His-tagged Tim23, tryptophan-requiring transformants could be obtained, confirming the functionality of the tagged Tim23. One of the transformants was chosen for further work and its mitochondrial protein profile in comparison to wild type is shown in Figure 10B.

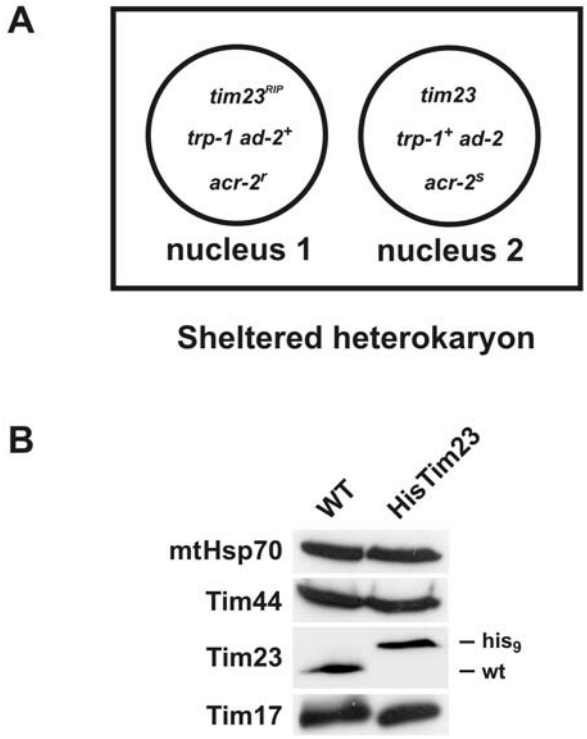

Figure 10. His-tagged Tim23 is functional in *N. crassa*

(**A**) Schematic presentation of the *tim23* null mutant in *N. crassa*. The heterokaryon was generated by the sheltered RIP method. (**B**) Sheltered heterokaryon in panel A was transformed with the plasmid carrying N-nonahistidinyl tagged Tim23. Tryptophan-requiring transformants were isolated and their mitochondria analyzed by SDS-PAGE and immunodecoration with the indicated antibodies in comparison to wild type (WT).

3.1.6. Stability of the TIM23 complex in *N. crassa*

Data obtained with yeast TIM23 complex showed that it is stable in digitonin but not in Triton X-100 or octylglucoside (Berthold *et al.*, 1995). Since digitonin is not a detergent of choice (it is a natural product with batch-to-batch variations, it is difficult to remove and it does not have defined physicochemical characteristics) about 15 different detergents were tested for the purification of the TIM23 complex. They are listed below:

-non-ionic: digitonin, dodecylmaltoside, Triton X-100, octylglucoside, $C_{12}E_6$, BigCHAP, deoxyBigCHAP, MEGA-8, TWEEN 20, BRIJ35 and n-heptyl-thioglucopyranoside

-ionic: CHAPS, CHAPSO, cholic acid, deoxycholic acid and SDS

Out of them all digitonin was the only one which did not destroy Tim17-Tim23 interaction. An example of these experiments is given in Figure 11. These experiments confirmed the previous finding from the coimmunoprecipitation experiments that Tim44 is not stably associated with Tim17-Tim23 subcomplex in *N. crassa* as it could not be copurified via the His-tag on Tim23. The specificity of Tim17 association is supported by the fact that none of the control proteins, such as Tim22, Oxa1, Tom40, mtHsp70 or AAC, was copurified.

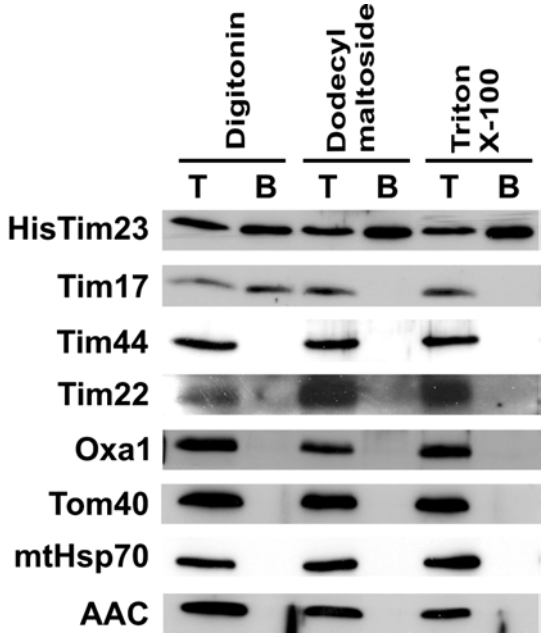

Figure 11. Stability of the TIM23 complex in *N. crassa*

Mitochondria containing His-tagged Tim23 were solubilized with the indicated detergents and extracts incubated with NiNTA-agarose beads. Beads were washed and bound material eluted with Laemmli buffer containing 300 mM imidazole. Samples were analyzed by SDS-PAGE and immunodecoration with antibodies indicated. T-total extract; B-material bound to the NiNTA-agarose beads.

3.1.7. Purification of the TIM23 complex

The purification strategy applied was to solubilize mitochondria isolated from a strain expressing an N-terminally His-tagged Tim23 with digitonin and purify the complex via a NiNTA-agarose column. Final purification was on an anion-exchange column. In preliminary experiments it became obvious that isolation of membranes prior to solubilization considerably increased the purity of the complex. Furthermore, phosphate buffer gave optimal binding to the NiNTA-agarose column but it had to be changed to TRIS prior to the ion-exchange chromatography. Typical purification procedure is given below. All steps were carried out at +4^{o}C.

Mitochondria (1 g) containing HisTim23 were resuspended at 10 mg/ml in buffer A (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM PMSF) and sonicated on ice for a total of 20 cycles each consisting of 12 s sonication and 18 s break (Branson sonifier, output 6, duty cycle 80%). Membrane fraction was isolated by ultracentrifugation in Ti70 rotor at 50000 rpm (250000 x g) for 45 min and resuspended in the starting volume of buffer A containing 2% (w/v) digitonin. After 45 min solubilization, nonsolubilized material was removed by ultracentrifugation in Ti70

rotor at 50000 rpm for 30 min and the solubilized fraction passed over 4 ml NiNTA-agarose column at a flow rate of 1 ml/min. The column was washed with 10 column volumes (CV) of buffer A containing 0.05% digitonin followed by 4 CV of buffer A containing 0.05% digitonin but NaCl concentration reduced to 10 mM. Bound material was eluted with the latter buffer containing 300 mM imidazole and immediately loaded on a PD-10 column (Amersham) equilibrated with buffer B (50 mM TRIS, pH 8.0, 10 mM NaCl, 10% glycerol, 0.05% digitonin). Protein-containing fractions eluted from the PD-10 column were loaded on a 1 ml Resource Q column (Amersham) at a flow rate of 0.5 ml/min using Äkta purifier system (Amersham). Bound material was eluted with a NaCl gradient in buffer B starting from 10 mM and ending at 500 mM in total of 10 CV.

An SDS-PA gel monitoring the various purification steps is given in Figure 12. The purified TIM23 complex contained 3 clearly visible protein bands. Tim17 and Tim23 were identified by immunoblotting and/or mass spectrometry. The protein of ca. 56 kDa represented a possibly new component of the complex and was therefore sent for mass spectrometric analysis (see below).

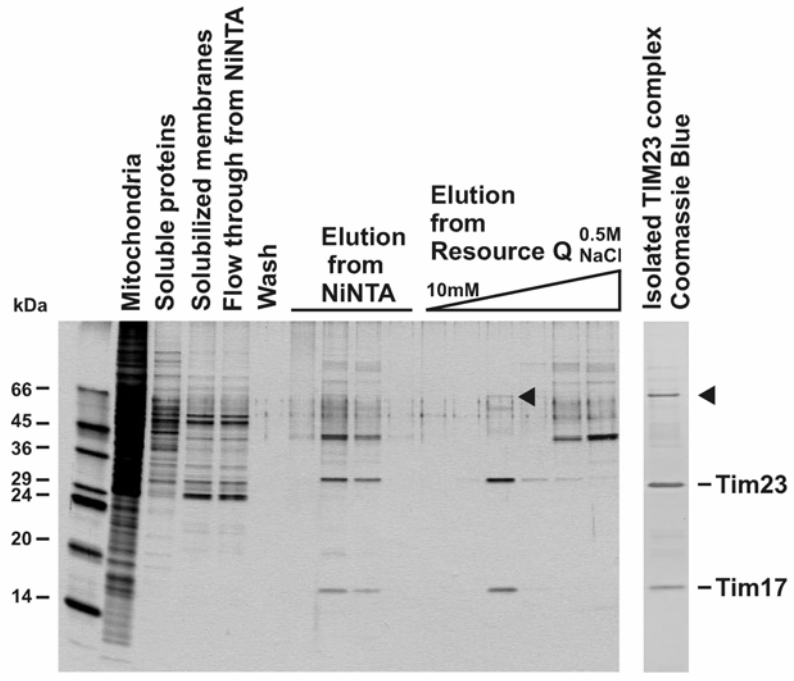

Figure 12. Purification of the *N. crassa* TIM23 complex

Mitochondrial membranes isolated from a strain expressing His-tagged Tim23 were solubilized with digitonin and the detergent extract was passed over a NiNTA-agarose column. Bound material was eluted with an imidazole-containing buffer and further purified on an anion-exchange column. Purification was monitored by SDS-PAGE and silver staining. Coomassie staining of the purified TIM23 complex is shown in the right panel. The 56 kDa protein is indicated by an arrow head.

3.2. Tim50 – a new component of the TIM23 complex

3.2.1. Identification of the 56 kDa protein copurifying with TIM23 complex

A mass spectrum obtained after trypsin digestion of the 56 kDa protein is shown in Figure 13. The fragments obtained were used to search the *N. crassa* database. The fingerprint after trypsin digestion matched the one deduced from the previously unidentified open reading frame (NCU02943.1).

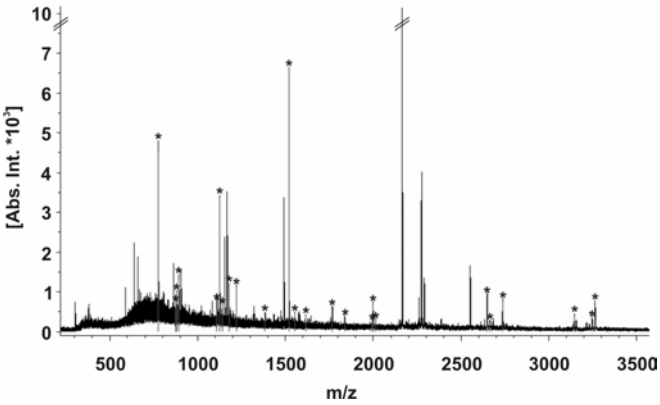

Figure 13. Mass spectrometric analysis of the 56 kDa protein present in the purified TIM23 complex

The protein band corresponding to an apparent molecular mass of about 56 kDa was excised from a Coomassie stained gel and subjected to trypsin digestion and analysis by mass spectrometry. The mass spectrum obtained is shown and the masses derived from peptides found in NCU02943.1 are marked with asterisks.

The NCU02943.1 reading frame codes for a protein consisting of 540 amino acid residues. It is predicted to contain a mitochondrial targeting sequence at the N terminus and an α-helical hydrophobic membrane spanning stretch between residues 171 and 191 (Figure 14). Cleavage of the targeting signal occurs either after residue 38, as the following amino acid is found at the N terminus of the purified protein, or more likely after residue 37, as this is the canonical MPP-cleavage site (Gavel and von Heijne, 1990). These structural features predict a protein anchored in the inner membrane with its N terminus located in the matrix and a large hydrophilic domain in the IMS.

Database searches revealed that its homologues are present in virtually all eukaryotes. Figure 14 shows the alignment of the *N. crassa* protein with the homologues from *S. cerevisiae*, *C. elegans* and *H. sapiens*. Like the *N. crassa* protein, they all contain an MTS, a predicted transmembrane domain and a large hydrophilic C-terminal domain.

Even though all structural elements are conserved, clear sequence homology is restricted to the C-terminal domain following the predicted transmembrane segment.

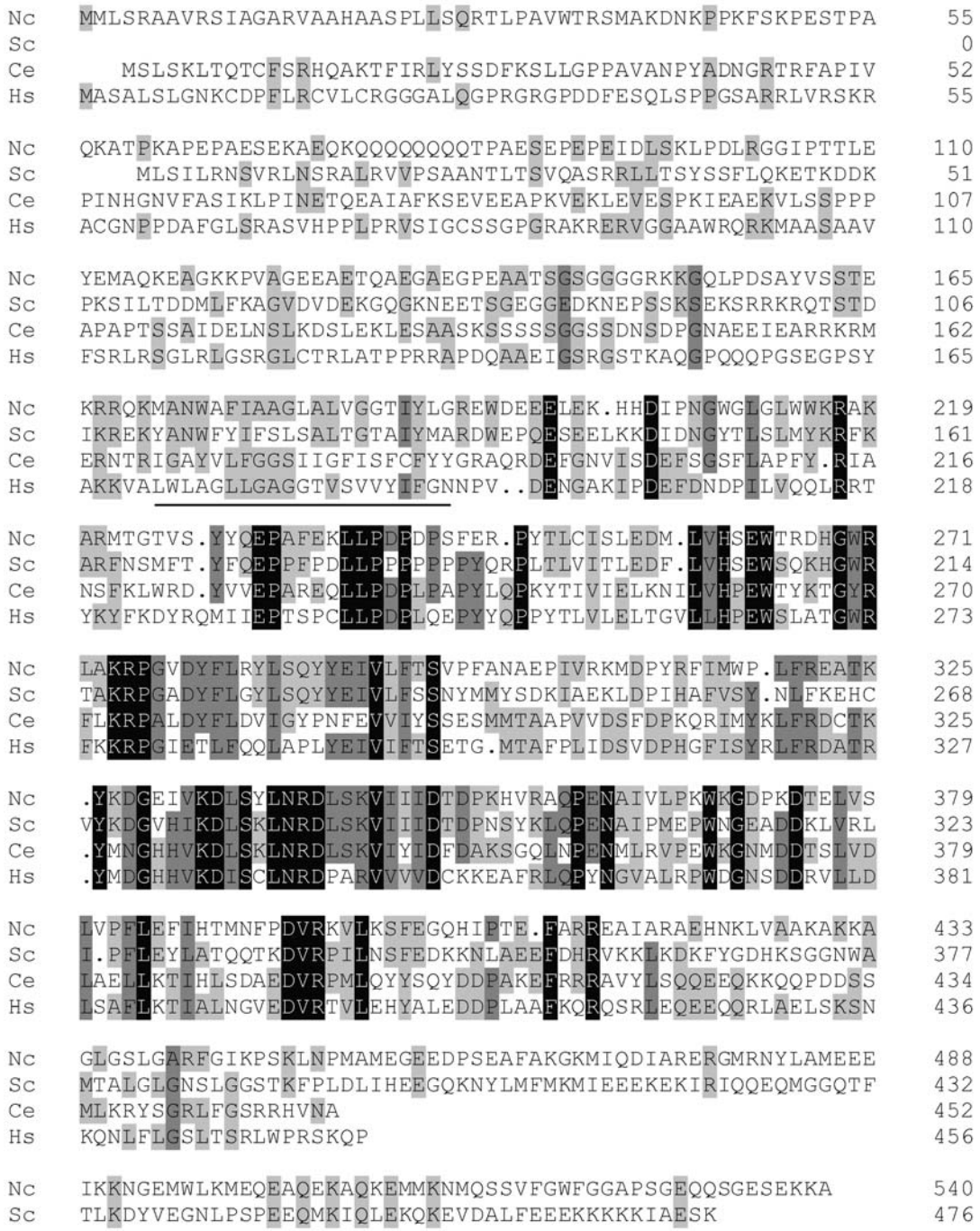

Figure 14. Alignment of the *N. crassa* 56 kDa protein with its homologues from *S.cerevisiae* (YPL063w), *C.elegans* (T25076) and *H.sapiens* (XM_053074)

Shaded and black backgrounds indicate partial and complete sequence conservation, respectively. The predicted single transmembrane domain is underlined.

3.2.2. Subcellular and submitochondrial localization of the 56 kDa protein

To verify the predicted topology of the 56 kDa protein, an antiserum was raised in rabbits against the hydrophilic C-terminal domain expressed in *E. coli* and affinity purified antibodies were used in subcellular and submitochondrial localization experiments. The 56 kDa protein was found in the mitochondrial fraction (Figure 15A). Digitonin fractionation of mitochondria revealed that the protein is accessible to protease only after opening of the outer membrane in agreement with the predicted removal of the large IMS domain (Figure 151B). Carbonate extraction experiment confirmed the presence of the TM domain (Figure 15C).

In summary, the 56 kDa protein which copurified with the TIM23 complex is an inner mitochondrial membrane protein with a large C-terminal domain exposed to the IMS.

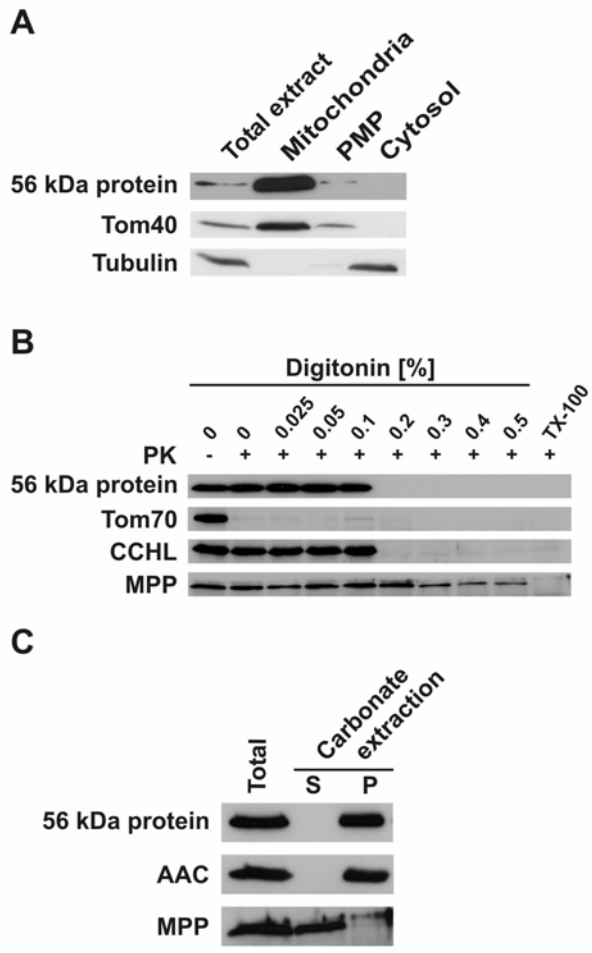

Figure 15. Subcellular localization of the 56 kDa protein

(**A**) Total *N. crassa* cell extract was subjected to differential centrifugation and fractions corresponding to mitochondria, postmitochondrial pellet (PMP) and cytosol were analyzed by SDS-PAGE and immunodecoration. Tom40 and tubulin were used as markers for mitochondria and cytosol, respectively. (**B**) Mitochondria were incubated with increasing concentrations of digitonin to successively open outer and inner membrane. Samples were treated with proteinase K (PK) and analyzed by SDS-PAGE and immunodecoration with antibodies against the 56 kDa protein, and Tom70, CCHL and MPP as markers for the outer membrane, intermembrane space and the matrix, respectively. (**C**) Mitochondria were treated with 0.1 M sodium-carbonate, and soluble (S) and membrane (P) fractions separated by centrifugation. Samples were analyzed as above using antibodies against AAC and MPP as controls for integral membrane and soluble proteins, respectively.

3.2.3. Interaction of the 56 kDa protein with the TIM23 complex

The 56 kDa protein copurified with the TIM23 complex in a large-scale purification, so is it really associated with the complex in a specific manner? Wild type *N. crassa* mitochondria were solubilized with digitonin and subjected to immunoprecipitation with antibodies to Tim17, Tim23, Oxa1 and the 56 kDa protein in a way that the corresponding antigen was immunodepleted from the detergent extract. Supernatants and pellets after immunoprecipitations were subjected to SDS-PAGE and analyzed by immunodecoration with the same antibodies as well as with an unrelated, abundant protein of the inner membrane (AAC) (Figure 16A). Antibodies to Tim17 virtually depleted both Tim17 and Tim23 from the supernatant. In addition, a significant amount of the 56 kDa protein was found in the immunoprecipitate. However, its larger portion was still in the supernatant. Antibodies to Tim23 gave an essentially identical result. Antibodies to the 56 kDa protein immunodepleted this protein from the extract and the immunoprecipitate contained significant amounts of both Tim17 and Tim23. Yet, their larger portions were in the supernatants. Oxa1 antibody depleted this protein from the extract and the immunoprecipitate contained none of the Tims or the 56 kDa protein. The high specificity of the coimmunoprecipitations was further supported by the fact that none of the immunoprecipitates contained AAC, one of the most abundant proteins of the inner membrane.

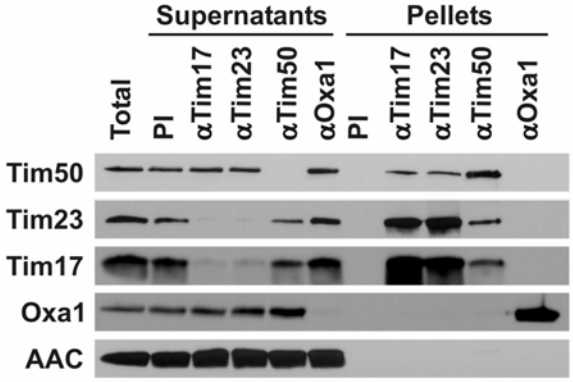

Figure 16. Coimmunoprecipitation of Tim50 with the TIM23 complex

Wild type *N. crassa* mitochondria were solubilized with digitonin and incubated with antibodies against Tim50, Tim17, Tim23 and Oxa1, or with preimmune IgGs prebound to protein A-sepharose beads. After collection of beads, supernatants were directly diluted with Leammli buffer. Beads were washed and bound proteins eluted with Laemmli buffer. Pellets and supernatants (representing 25% of the material present in pellets) were analyzed by SDS-PAGE and immunodecoration.

In the second set of experiments, mitochondria containing His-tagged versions of the 56 kDa protein, Tim23, Tim22 or Oxa1 were solubilized with digitonin and incubated with NiNTA-agarose beads. After washing steps, bound proteins were eluted with Laemmli buffer containing 300mM imidazole and analyzed by SDS-PAGE and immunodecoration with the depicted antibodies (Figure 17). When mitochondria containing His-tagged 56 kDa protein were used, both Tim17 and Tim23 were partly retained on the beads, along with 56 kDa protein. With mitochondria containing His-tagged Tim23, Tim17 and Tim23 were retained as well as part of the 56 kDa protein. None of the control proteins such as Oxa1, Tom40 or AAC were bound the beads confirming the specificity of the interactions. Furthermore, when control strains expressing His-tagged Oxa1 or Tim22 were used, neither the 56 kDa protein nor any of the TIM23 proteins was retained on the beads.

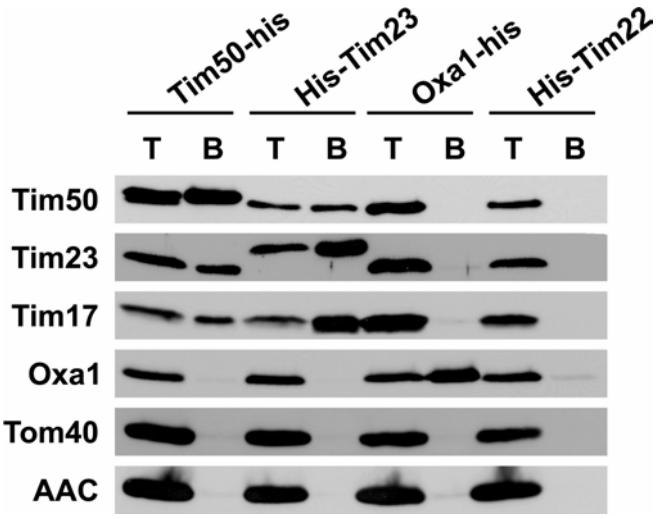

Figure 17. Copurification of Tim50 with the TIM23 complex and vice versa via NiNTA-agarose beads

Mitochondria were isolated from *N. crassa* strains in which either Tim50, Tim23, Oxa1 or Tim22 carried a His-tag and solubilized with digitonin. Detergent extracts were incubated with NiNTA-agarose beads and, after washing steps, bound proteins eluted with imidazole-containing buffer. Samples were analyzed by SDS-PAGE and immunodecoration. T, total solubilized material, representing 25% of input; B, material bound to the NiNTA-agarose.

These experiments demonstrate that the 56 kDa protein is indeed specifically associated with the TIM23 complex. Following the established nomenclature for the Tom and Tim

proteins (Pfanner *et al.*, 1996), the protein was named Tim50, according to the molecular weight of the yeast homologue.

3.2.4. Stoichiometries in the TIM23 complex

Only relatively minor amounts of Tim50 seem to be associated with the TIM23 complex, at least under these experimental conditions. In order to determine the percentage of total Tim50 associated with the TIM23 complex, *N. crassa* cells were metabolically labeled with ^{35}S sulphate and isolated mitochondria were subjected to coimmunoprecipitations as described above. Samples were subjected to SDS-PAGE and radioactive signals in protein bands quantified using a phosphorimager. About 10-20% of total Tim50 was found associated with Tim17 and Tim23, as judged by the Tim50 signal intensities in immunoprecipitates with Tim17 and Tim23 antibodies compared to the one obtained with Tim50 antibodies. Relative total amounts of these three proteins were determined after immunoprecipitation from SDS-solubilized mitochondria. Considering the specific contents of sulphur-containing amino acids, the molar ratio of Tim50:Tim23:Tim17 was determined to be ca. 2:1:1 (Figure 18). Thus the molar ratio in the purified complex was ca. 0.3:1:1. This reflects either a dynamic or labile interaction of Tim50 with the other two components.

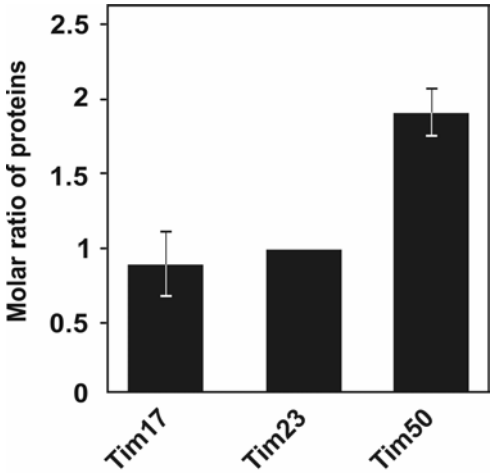

Figure 18. Quantification of Tim17, Tim23 and Tim50 in mitochondria

N. crassa cells were metabolically labeled with ^{35}S and mitochondria were prepared. They were solubilized with SDS and, after dilution with Triton X-100, subjected to quantitative immunoprecipitation with affinity purified antibodies against Tim17, Tim23 and Tim50. Immunoprecipitates were analyzed by SDS-PAGE and phosphorimaging. To obtain molar ratios, signals were corrected for differences in radioactivity incorporated into Tim17 (7 S), Tim23 (6 S) and Tim50 (19 S). The amount of Tim23 was set to 1. Error bars represent the standard error from five independent experiments.

3.2.5. Tim50 interacts with precursors in the intermembrane space

Genetic analysis in yeast showed that Tim50 is encoded by an essential gene (Mokranjac *et al.*, 2003a). The same observation was made in *N. crassa* (S. Hoppins and F. Nargang, unpublished results). Import experiments using yeast mitochondria selectively depleted of Tim50 demonstrated that it has a distinct role in the import of preproteins along the TIM23 pathway (Mokranjac *et al.*, 2003a). In order to understand the role of Tim50 in protein import into mitochondria, its interactions with preproteins arrested at different stages of translocation were analyzed by crosslinking. A precursor protein, pb_2Δ19(167)DHFR$_{K5}$ (see section 3.1.3.), was incubated with wild type yeast mitochondria under the following conditions: a) in the absence of a membrane potential, when the precursor is partially translocated across the outer membrane but does not reach the channel in the inner membrane; b) in the presence of a membrane potential, when the precursor protein is completely translocated into the matrix and c) in the presence of a membrane potential and methotrexate, added to stabilize the folded DHFR domain, when the N terminus of the protein reaches the matrix but the DHFR domain is still outside of mitochondria so that the protein is arrested as an intermediate spanning both mitochondrial membranes. Crosslinking experiments with a very short crosslinker DFDNB (0.3 nm) were performed under each of these conditions and samples subjected to immunoprecipitations under stringent conditions with antibodies to Tim50 and various other known import components. Interestingly, Tim50 was crosslinked already to the precursor partially translocated across the outer membrane, in contrast to all other TIM23 components tested (Figure 19, top panel). An intermediate spanning both membranes was still crosslinked to Tim50 but now, in addition, crosslinks to Tim23, Tim44 and mtHsp70 appeared (Figure 19, middle panel). Precursor completely translocated into the matrix was found in contact only with mtHsp70 (Figure 19, lower panel). These experiments demonstrate that Tim50 interacts with precursors or at least is in their close vicinity in the IMS. Notably, the crosslinking pattern of Tim50 to precursor partially translocated across the outer membrane was quite different from the pattern observed with the intermediate spanning both membranes. This most likely represents different, dynamic interactions of Tim50 with precursors at various stages of the import process.

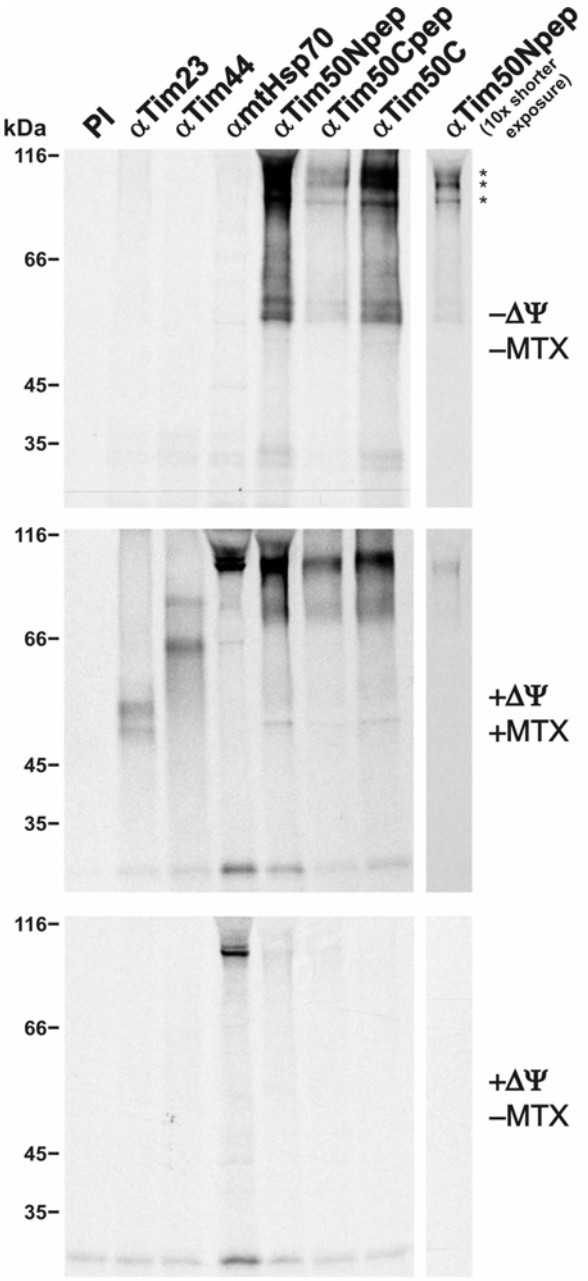

Figure 19. Crosslinking of Tim50 to the preprotein in transit

Precursor pcytb_2Δ19(167)DHFR$_{K5}$ was imported into yeast mitochondria in the way that it is arrested at the level of the outer membrane (top panel), as an intermediate spanning both outer and inner membrane (middle panel) and completely imported into the matrix (lower panel). Samples were subjected to crosslinking with DFDNB and, after quenching of the crosslinker, subjected to immunoprecipitations under stringent conditions with the indicated antibodies. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. PI-preimmune serum; Asterisks in the top panel indicate observed crosslinks to Tim50.

In order to confirm such a dynamic interaction between the translocating precursor and Tim50, the precursor protein was first accumulated without a membrane potential and then, upon establishment of a membrane potential, chased into the matrix. The crosslinking to Tim50 and translocation across the inner membrane were followed in a time-dependent manner. Crosslinked species were observed at a zero time point and, upon establishment of ΔΨ, their intensities gradually decreased (Figure 20, upper panel). At the same time, precursor was translocated into the matrix and processed by MPP (Figure 20, lower panel). The kinetics of import into the matrix corresponded to the decrease of

crosslinking to Tim50. This suggests that Tim50 is in contact with the precursor as soon as it reaches the *trans* side of the TOM complex and as long as its segments are present in the IMS.

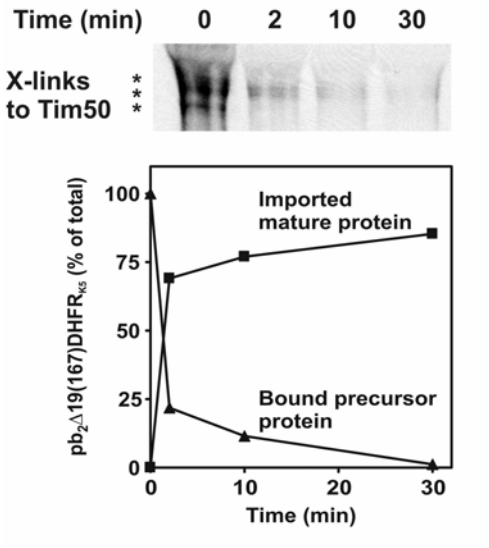

Figure 20. Precursor bound to Tim50 in the absence of membrane potential is a productive translocation intermediate

Precursor pcytb_2Δ19(167)DHFR$_{K5}$ was bound to mitochondria in the absence of membrane potential. Mitochondria were reisolated, re-energized and incubated further. At time points indicated, aliquots were withdrawn for crosslinking or for SDS-PAGE directly. Upper panel: samples were subjected to crosslinking and immunoprecipitations with Tim50 antibodies before analysis by SDS-PAGE and autoradiography. Lower panel: samples were directly subjected to SDS-PAGE and analyzed by autoradiography and quantification. The amounts of bound precursor and imported mature protein at various time points were related to the total precursor bound at time point zero which was set to 100%.

Crosslinks of Tim50 to precursors bound to mitochondria without membrane potential showed for the first time that precursors reach the TIM machinery under these conditions. It was therefore important to check if this behavior is somehow specific for the precursor used or it is a general characteristic of the TIM23 substrates. Various TIM23 substrates, targeted to the matrix or to the inner membrane via a stop-transfer mechanism or in a conservative manner, were bound to mitochondria without membrane potential and their interactions to Tim50 analyzed by crosslinking and immunoprecipitation (Figure 21). Specific crosslinking adducts of Tim50 to all precursors tested were obtained showing that Tim50 is indeed the first component of the TIM23 translocase which gets in contact with precursors once they emerge from the TOM complex. Furthermore, Tim50 is apparently able to recognize all the various classes of TIM23 substrates. It is therefore tempting to speculate that the major function of Tim50 in import into mitochondria is to

recognize the TIM23 substrates which are appearing at the outlet of the TOM complex and guide them to the import channel in the inner membrane.

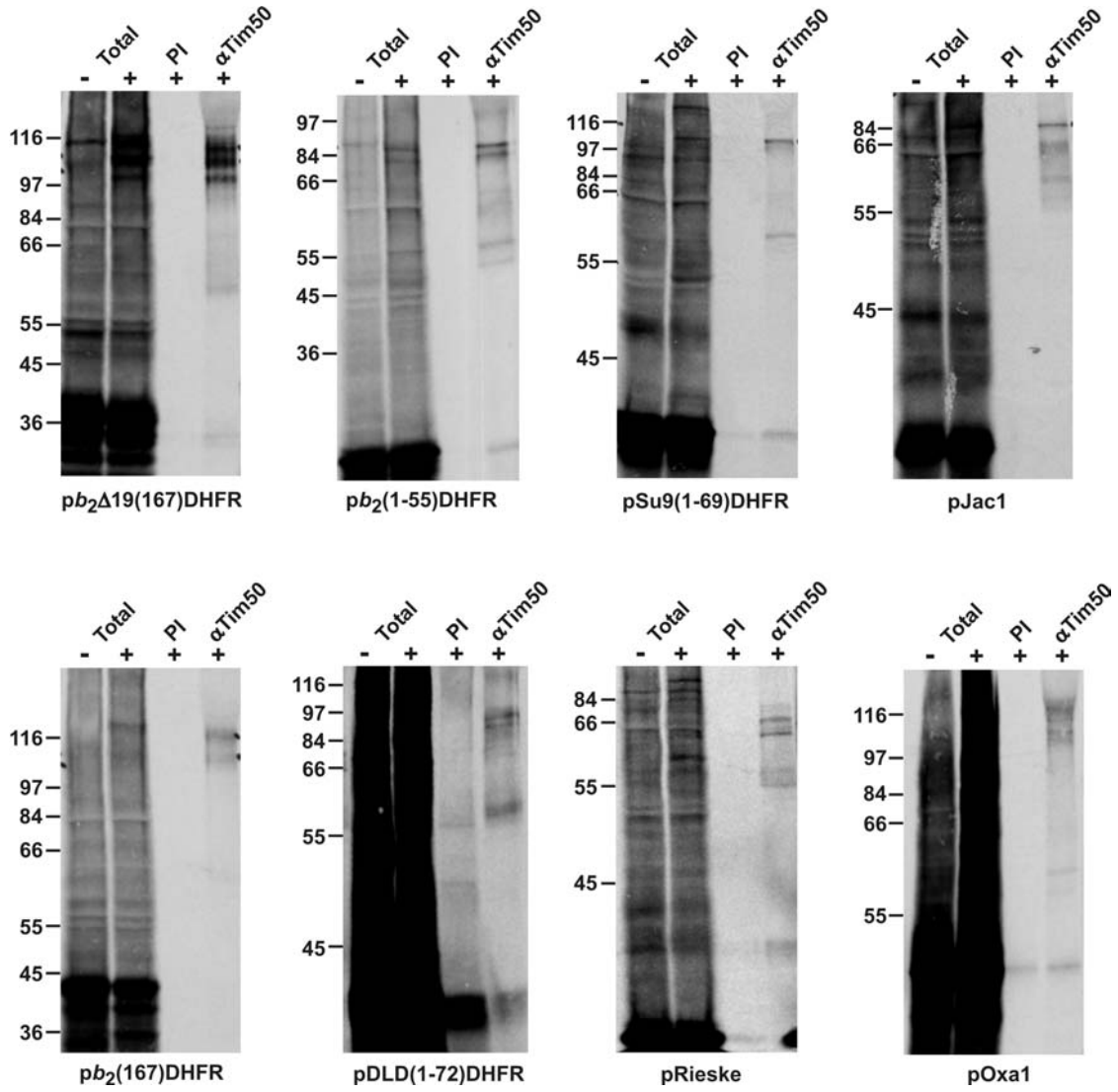

Figure 21. Tim50 recognizes all classes of TIM23 substrates

Various TIM23 substrates were bound to mitochondria without membrane potential. Samples were mock treated (-) or subjected to crosslinking with DFDNB (+) and either directly analyzed by SDS-PAGE and autoradiography or first immunoprecipitated with Tim50 antibodies. Preimmune serum (PI) was used as a control.

3.3. Tim14 – the J-protein in the import motor of the TIM23 translocase

3.3.1. Tim44 is in the neighborhood of an unidentified protein

Purification trials of the *N. crassa* TIM23 complex have revealed its highly unstable nature. Still, its purification led to the identification of Tim50, the new component with an essential role in protein import into mitochondria. Thus, the possibility existed that some components have escaped detection, especially concerning the fact that Tim44, the known component of the complex, was not found in the purified complex. Indeed, the presence of several, so far unknown components has repeatedly been implicated in the literature (Berthold *et al.*, 1995; Blom *et al.*, 1995; Moro *et al.*, 1999). In particular, a crosslink of Tim44 to an unidentified protein of about 15 to 20 kDa was reported (Moro *et al.*, 1999). This observation was confirmed (Figure 22). An approach was taken to identify this component by *in silico* analysis. The availability of the sequenced genome of yeast (Goffeau *et al.*, 1996), as well as several genome-wide analyses dealing with viability of deletion mutants (Giaever *et al.*, 2002) (Winzeler *et al.*, 1999) and subcellular localizations of the yeast proteome (Kumar *et al.*, 2002), combined with the possibility to predict the presence of MTS (Claros and Vincens, 1996) have created a powerful and a straightforward way to identify essential genes of unknown functions which are possibly localized in mitochondria. Of course, mitochondrial proteins without classical MTS would still escape detection unless they were localized to mitochondria in the high throughput screen.

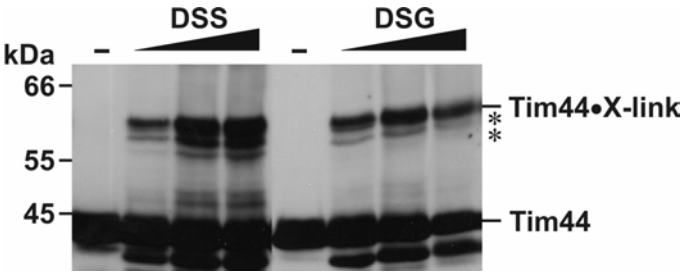

Figure 22. Yeast Tim44 is in the neighborhood of an unidentified protein of ca. 15-20 kDa

Wild-type yeast mitochondria were subjected to crosslinking with DSS and DSG. After quenching of crosslinker, samples were analyzed by SDS-PAGE and immunodecoration with antibodies to Tim44. Minor crosslinks are labeled with asterisks.

In order to identify the protein following four criteria were used for database searches:

1) its molecular weight should be between 15 and 20 kDa,
2) it should have a mitochondrial targeting signal,
3) it should be encoded by an essential gene,
4) it should have homologues throughout the eukaryotic kingdom.

ORF *YLR008c* turned out to be a candidate. It fulfilled all the above mentioned criteria and furthermore it contained a J-domain, known for its stimulatory activity on the Hsp70 class chaperones.

To verify that this is indeed the protein which Tim44 could be crosslinked to, a yeast strain harboring an octahistidinyl tag at the N terminus of the chromosomal copy of *YLR008c* was constructed and used to check if the particular crosslinking adduct of Tim44 could be isolated via NiNTA-agarose beads. Mitochondria isolated from this strain as well as from wild type yeast strain were subjected to crosslinking with DSS and, after quenching of the crosslinker, samples were split. One part was directly dissolved in Laemmli buffer for SDS-PAGE analysis. The other part was solubilized in SDS-containing buffer to destroy all protein-protein interactions and, after dilution with Triton X-100 buffer and a clarifying spin, incubated with NiNTA-agarose beads to bind all His-tag containing proteins and crosslinking adducts. After three washing steps, bound proteins were eluted with Laemmli buffer containing imidazole and all samples were subjected to SDS-PAGE and immunodecoration with Tim44 antibodies (Figure 23). The overall crosslinking patterns in both types of mitochondria were virtually identical, however the ca. 60 kDa crosslinking adduct of Tim44 was bound to the NiNTA-agarose beads only with mitochondria containing His-tagged version of *YLR008c*. Obviously, the right protein was identified. It was named Tim14, according to the above mentioned nomenclature.

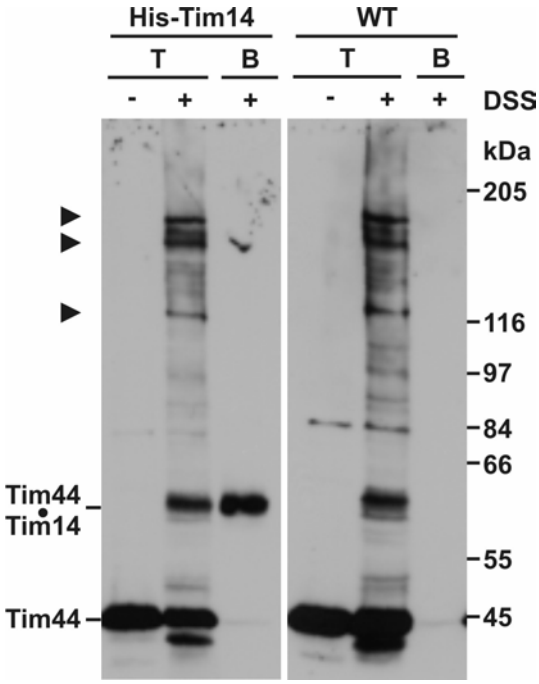

Figure 23. Tim14 is encoded by the *YLR008c* reading frame

Mitochondria were isolated from a yeast strain in which a sequence encoding eight histidines was fused to the 5´end of the *YLR008c* reading frame, and from a corresponding wild type. Both types of mitochondria were subjected to crosslinking with DSS. After quenching of the crosslinker, one aliquot of each type of mitochondria was directly analyzed by SDS-PAGE; the other aliquot was solubilized and incubated with NiNTA-agarose beads. Bound material was eluted and subjected to SDS-PAGE. Samples were analyzed by immunodecoration with Tim44 antibodies. T-total mitochondria incubated without or with DSS; B-material bound to NiNTA-agarose beads; Arrowheads indicate crosslinking adducts of Tim44 to mtHsp70.

3.3.2. Localization of Tim14 in mitochondria

Antibodies to Tim14 were generated in rabbits using a His-tagged recombinant protein consisting of amino acids 50 to 168 of Tim14 as an antigen. Affinity purified antibodies recognized a single band in total yeast cell extract and in isolated mitochondrial proving exclusive mitochondrial location of Tim14 (Figure 24A.)

In order to localize Tim14 within mitochondria, its accessibility to the externally added protease in intact mitochondria, mitoplasts or completely solubilized mitochondria was analyzed (Figure 24B). Tim14 was protected against protease in intact mitochondria but in mitoplasts it was degraded to a specific fragment. Upon addition of Triton X-100 in order to solubilize both mitochondrial membranes, Tim14 was completely degraded. The fragment obtained in mitoplasts was the C-terminal one as it could be decorated with an HA-specific antibody when the same experiment was done using mitochondria containing C-terminally HA-tagged Tim14. Upon carbonate extraction of mitochondria, Tim14 partly fractionated with the membranes, the larger part was, however, found in the

supernatant. Tim14 apparently belongs to the class of inner membrane proteins which span the membrane once but do not firmly interact with membrane lipids.

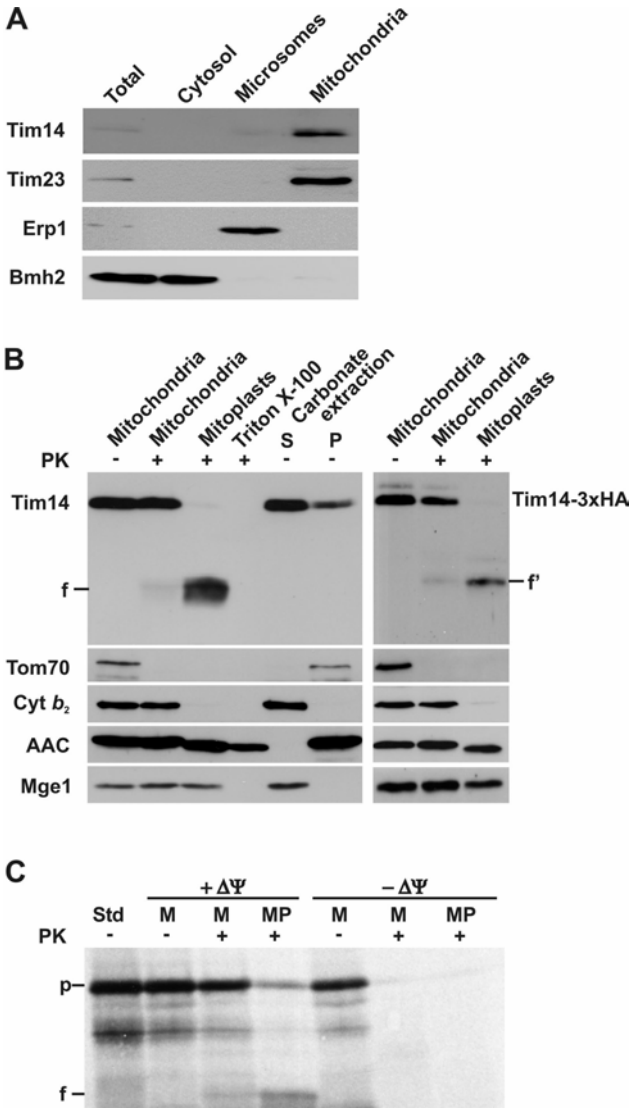

Figure 24. Tim14 is an inner mitochondrial membrane protein exposing its C terminus into the matrix

(**A**) Equal amounts of protein of subcellular fractions were subjected to SDS-PAGE and immunodecoration with antibodies against Tim14 and marker proteins of mitochondria (Tim23), microsomes (Erp1) and cytosol (Bmh2). (**B**) The submitochondrial location of Tim14 was analyzed by its accessibility to the externally added proteinase K (PK) in intact mitochondria, in mitoplasts or in completely solubilized mitochondria. Membrane association of Tim14 was analyzed by carbonate extraction. The behavior of Tim14 was compared to the marker proteins of various subcompartments. f, 13 kDa fragment of Tim14. Right panel: Mitochondria containing a 3xHA tag at the C terminus of Tim14 were analyzed in the same way, this time following the HA tag. f', fragment of Tim14. (**C**) ^{35}S-labeled Tim14 was synthesized in the reticulocyte lysate and incubated with mitochondria in the presence or absence of membrane potential ΔΨ. Mitochondria were reisolated, aliquots converted to mitoplasts and treated with PK. Samples were subjected to SDS-PAGE and autoradiography. M, mitochondria; MP, mitoplasts; Std, 40% of input into import experiments; p, precursor of Tim14; f, 13 kDa fragment.

Mitochondrial location of Tim14 and presence of a mitochondrial targeting signal in it were further supported by the fact that the protein could be posttranslationally imported into isolated mitochondria after synthesis in reticulocyte lysate (Figure 24C). Radioactively labeled protein was imported into protease protected location within mitochondria in a membrane potential-dependent manner. Upon swelling of mitochondria, imported protein was converted to the same fragment as the endogenous protein.

In conclusion, Tim14 is anchored in the inner mitochondrial membrane by a single transmembrane domain and it exposes its C terminus into the matrix.

3.3.3. Tim14 is a J-domain protein essential for viability of yeast

The amino acid sequence of Tim14, as deduced from the DNA sequence of *YLR008c*, is shown in Figure 25. Tim14 contains an N-terminal hydrophilic segment of 65 amino acid residues followed by a predicted α-helical transmembrane domain of 18 residues and a hydrophilic C terminal domain of 85 residues. This C-terminal segment contains a domain of ca. 50 residues with strong similarity to the J-domain of DnaJ-like proteins (Bukau and Horwich, 1998; Kelley, 1998). Thus, the J-domain of Tim14 is at the matrix side of the inner membrane.

Why is the J-domain so important? DnaJ is a cochaperone of *E.coli* responsible for the stimulation of the ATPase activity of DnaK, bacterial Hsp70 (Kelley, 1998). This stimulatory function was narrowed down to its N-terminal domain known as the J-domain (Liberek *et al.*, 1991; Misselwitz *et al.*, 1998; Szabo *et al.*, 1994; Wall *et al.*, 1994). DnaJ relatives, either full length homologues or the ones containing just the J-domain, are present in practically all prokaryotes and eukaryotes (Kelley, 1998). In mitochondria, three DnaJ relatives have been described so far but none of them has a role in the import process (Kim *et al.*, 2001; Rowley *et al.*, 1994; Westermann and Neupert, 1997). It was therefore suggested that Tim44 has taken over this function even though it does not have a J-domain (Merlin *et al.*, 1999). Identification of Tim14 raised the exciting possibility that mtHsp70 does after all need a stimulatory factor, and that this function is fulfilled by Tim14.

All eukaryotes apparently have Tim14 orthologs which share three common elements:

1) a highly conserved C-terminal domain which includes the J-domain,
2) a transmembrane domain preceding it, and

3) a putative mitochondrial targeting signal directly after the transmembrane domain.

Figure 25 shows the alignment of Tim14 with its homologues from *S. pombe*, *C. elegans* and *H. sapiens*. There are two closely related proteins with the above characteristics in humans.

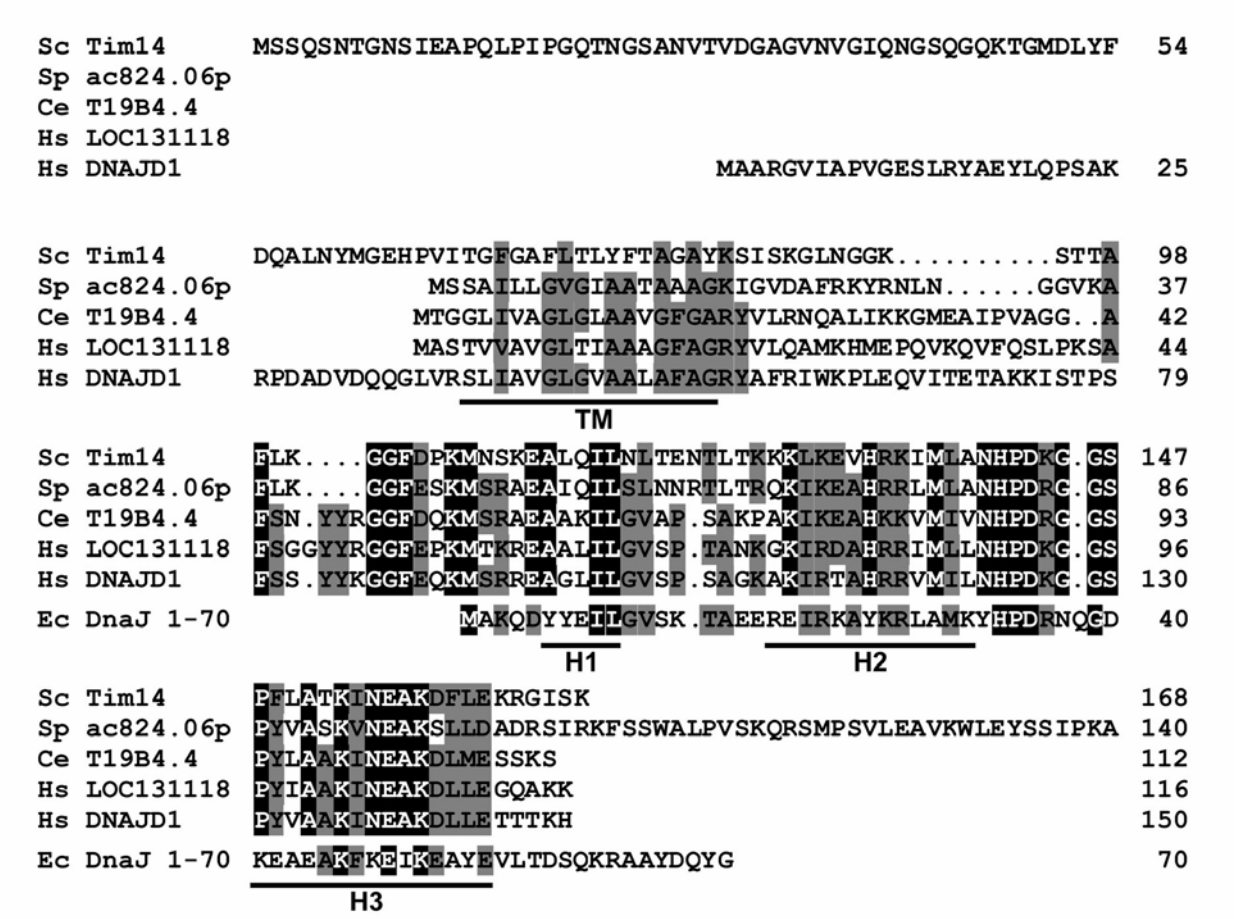

Figure 25. Alignment of Tim14 proteins from different organisms and the J-domain of *E.coli* DnaJ

Sc, *S. cerevisiae*; Sp, *S. pombe*; Ce, *C. elegans*; Hs, *H. sapiens*; Ec, *E. coli*; Single predicted transmembrane domain (TM) is underlined. Black and grey shadings represent identical and similar residues, respectively. H1-H3, characteristic α-helical segments of the J-domain of DnaJ.

The *TIM14* gene was deleted in diploid cells and these were subjected to tetrad analysis. Only two out of four spores were viable (Figure 26A) confirming the previous result from high throughput screens that *TIM14* is a gene essential for viability of yeast.

In order to generate a tool to study the function of Tim14, *TIM14* was placed under the control of the *GAL10* promoter. This yeast strain grew like wild type in the presence of galactose; however in its absence, i.e. in the presence of glucose, cells slowed down in their growth ca. 12 h after the shift and virtually stopped growing after ca. 26 h (Figure 26B). Mitochondria were isolated from this strain, as well as from the isogenic wild type, 21 h after the shift to a glucose-containing medium and levels of various mitochondrial proteins were determined by immunoblotting. Tim14 itself was virtually absent from these mitochondria (Figure 26C). In contrast, all other known components of the TIM23 translocase, Tim50, Tim17, Tim23, Tim44, mtHsp70 and Mge1, were present at wild type levels. Furthermore, Tim22 and Tim10 of the TIM22 translocase as well as Tom22 and Tom40 of the TOM complex were not affected by depletion of Tim14. Likewise, subunit 2 of the cytochrome oxidase complex, Cox2, $F_1\beta$ subunit of the ATP synthase, Mdj1 and the ADP/ATP carrier (AAC) were all present at control levels. This shows that depletion of Tim14 did not significantly affect the overall integrity of mitochondria.

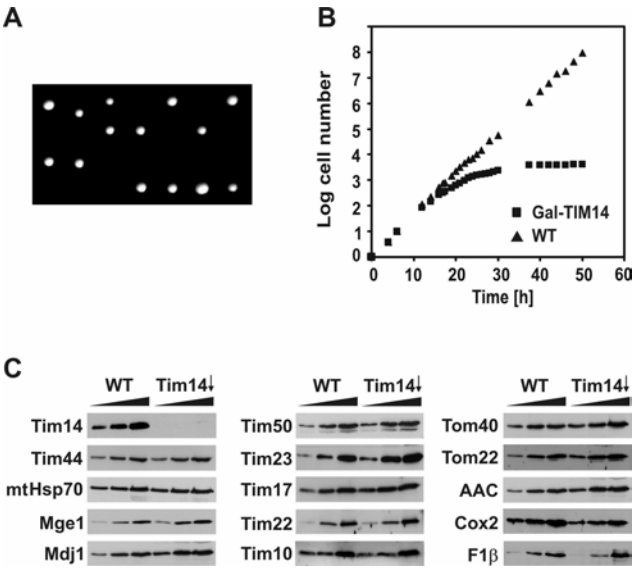

Figure 26. Tim14 is essential for yeast cell viability

(**A**) Tetrad analysis of diploid yeast strain carrying a deletion of *TIM14*. (**B**) Growth curve of a yeast strain carrying *TIM14* under *GAL10* promoter (GAL-TIM14) on glucose containing medium, and of a wild type strain (WT) for comparison. Cells were grown on galactose containing medium for 2 days before they were shifted to glucose. Number of cells at the time of the shift was set to 1. (**C**) Wild type cells and cells harboring *TIM14* under galactose control were collected 21 h after shift to glucose containing medium. Mitochondria were isolated and analyzed by SDS-PAGE and immunodecoration with the depicted antibodies.

3.3.4. Tim14 is required for import of preproteins

The majority of known mitochondrial proteins essential for viability of yeast have a distinct role in the topogenesis of mitochondrial proteins. Furthermore, the fact that Tim14 is in the neighborhood of Tim44 would strongly support its role in the mitochondrial import process. Therefore, import efficiencies into mitochondria isolated from cells depleted of Tim14 and from wild type cells as a control were compared. Various precursor proteins were tested. All tested matrix targeted proteins, which are using the TIM23 translocase, were imported into Tim14 depleted mitochondria with severely reduced efficiencies. Examples shown are pcytb_2Δ19(167)DHFR, a matrix targeted protein consisting of the first 167 residues of cytochrome b_2 fused to DHFR (with inactivated sorting signal) (Figure 27A), pF_1β, the precursor of β subunit of the F_1ATPase (Figure 27B) and pSu9(1-69)DHFR, a precursor consisting of the targeting signal of *N. crassa* subunit 9 of the F_oATPase fused to DHFR (Figure 27C, upper panel). In addition, this last precursor was imported as the recombinant protein in amounts that saturate the import system. In this case drastically reduced import efficiency was observed as well (Figure 27C, lower panel). Furthermore, a precursor of an inner membrane protein which uses the TIM23 translocase, pCoxVa, was strongly affected in its import (Figure 27D). In contrast, import of precursors which use the TIM22 translocase, such as AAC (Figure 27E) and Tim23 (Figure 27F), was not or only slightly affected. The same is true for precursors which use only TOM complex but none of the TIM translocases, such as cytochrome c heme lyase, CCHL (Figure 27G). Therefore, Tim14 is specifically needed for import of precursors along the TIM23 pathway.

If Tim14 is indeed an import component it should be in the proximity of the translocating chain. Therefore, pcytb_2Δ19$(DHFR)_{K5}$ was arrested as a both-membranes-spanning intermediate (see 3.1.3) and its environment probed by crosslinking with either DSS or DFDNB. One of the crosslinks obtained by DSS crosslinking could be specifically precipitated with affinity purified Tim14 antibodies (Figure 28) showing that Tim14 is at least in the close neighborhood of the translocating chain or might even interact with it.

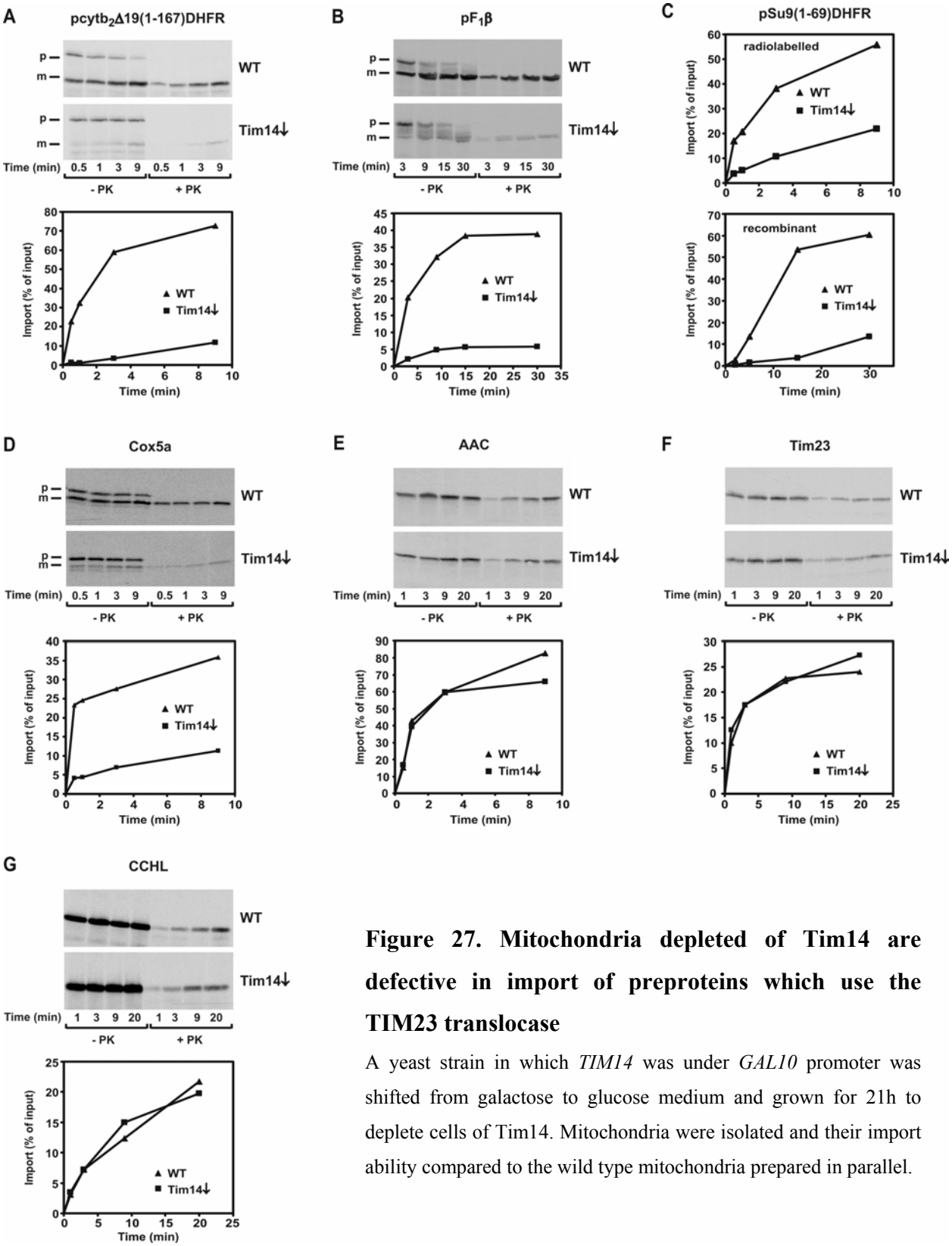

Figure 27. Mitochondria depleted of Tim14 are defective in import of preproteins which use the TIM23 translocase

A yeast strain in which *TIM14* was under *GAL10* promoter was shifted from galactose to glucose medium and grown for 21h to deplete cells of Tim14. Mitochondria were isolated and their import ability compared to the wild type mitochondria prepared in parallel.

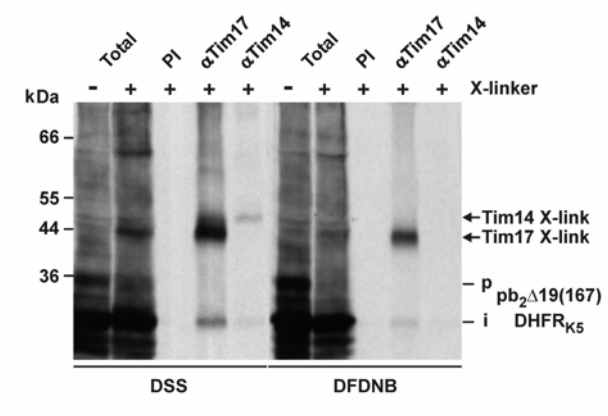

Figure 28. Tim14 is in the proximity of the translocating chain

^{35}S-labeled precursor pcytb_2Δ19(DHFR)$_{K5}$ was arrested in mitochondria as an intermediate spanning both membranes. After crosslinking with DSS or DFDNB, samples were subjected to immunoprecipitation under stringent conditions with antibodies to Tim17 and Tim14 and analyzed by SDS-PAGE and autoradiography.

3.3.5. Tim14 is part of the TIM23 complex

Tim14 was found as a protein which could be crosslinked to Tim44 and it has a specific role in the translocation via the TIM23 translocase, so is it also present in this complex? Antibodies to Tim17 and Tim23 were used in coimmunoprecipitation experiments to check the association of Tim14 with the known components of the TIM23 complex (Figure 29). When wild type mitochondria were solubilized with digitonin, both Tim17 and Tim23 antibodies efficiently co-precipitated Tim14 as well as Tim50 and Tim44 (Figure 29A). In order to get a better understanding of the structural organization of the TIM23 translocase, the same experiment was performed with mitochondria specifically depleted of one of its components. When mitochondria depleted of Tim17 were used, neither Tim14 nor Tim44 could be co-precipitated using either Tim23 or Tim17 antibodies (Figure 29B). Essentially the same result was obtained with mitochondria depleted of Tim23. Interestingly, Tim50 can still form a subcomplex with Tim23 in the absence of Tim17. With Tim44-depleted mitochondria, both Tim17 and Tim23 antibodies precipitated Tim17-Tim23-Tim50 subcomplex, but only minor amounts of Tim14, together with residual amounts of Tim44 (Figure 29C). Finally, when mitochondria depleted of Tim14 were used (Figure 29D), both Tim44 and Tim50 were associated with Tim17 and Tim23 as in the wild type.

These experiments clearly demonstrate that Tim14 is indeed an integral component of the TIM23 translocase and that it is recruited to the complex via Tim44. Furthermore, they suggest that Tim44 is able to stably interact only with the assembled Tim17-Tim23 subcomplex.

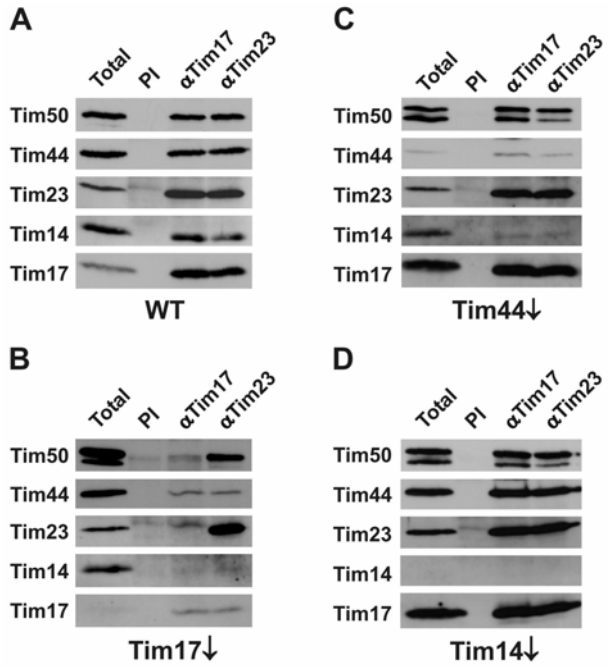

Figure 29. Tim14 is part of the TIM23 complex

Mitochondria from wild type yeast cells (**A**), Tim17-depleted cells (**B**), Tim44-depleted cells (**C**) and Tim14-depleted cells (**D**) were solubilized with digitonin and subjected to immunoprecipitations using Tim17 and Tim23 antibodies prebound to protein A-sepharose beads. Preimmune serum was used as a control. Beads were washed and bound proteins eluted with Laemmli buffer. Samples were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies.

3.3.6. Tim14 is a constituent of the import motor

The interaction of Tim14 with Tim44 and the presence of the J-domain strongly suggest that Tim14 has a function in the import motor. This function would imply dynamic interactions with other components of the motor in the course of its ATP-driven cycles. ATP-dependent association of mtHsp70 with Tim44 is well established (Neupert and Brunner, 2002; Pfanner and Geissler, 2001). In this work, crosslinking in intact mitochondria was used as a way to monitor the ATP-dependence of various interactions as this introduces fewer disturbances into the system compared to commonly used co-immunoprecipitation. Wild type mitochondria were preincubated at 25^{o}C with apyrase and oligomycin to deplete ATP or with NADH, ATP, creatine-phosphate and creatine-kinase to keep the ATP levels high. Samples were then crosslinked on ice with DSS and, after quenching of the crosslinker, analyzed by SDS-PAGE and immunodecoration with Tim44 antibodies (Figure 30). The same experiment was done with Tim14 depleted mitochondria, Figure 30. In wild type mitochondria, Tim44 interacted with mtHsp70 only at high ATP levels in the matrix. Upon ATP depletion, crosslinks of Tim44 to mtHsp70 disappeared. This is in agreement with previous results from coimmunoprecipitation experiments (Schneider *et al.*, 1994). Interestingly, the crosslink which was identified in

this study as a crosslink of Tim44 to Tim14 behaved in an opposite manner. It was weak at high ATP levels but became the most prominent crosslink of Tim44 in ATP-depleted mitochondria. When Tim14 depleted mitochondria were analyzed in the same experiment it became obvious that the Tim44 crosslinking pattern was completely different. Crosslinks of Tim44 to Tim14 were, of course, absent but few crosslinks of slightly smaller sizes appeared. The most obvious difference was the absence of crosslinks between Tim44 and mtHsp70. Instead a very strong crosslink of ca. 100 kDa appeared. It possibly represents a Tim44 dimer but this remains to be proven. It should be noted that disappearance of the Tim44-mtHsp70 crosslinks most likely reflects a conformational change as the ATP-dependent complex of these two proteins could still be detected in coimmunoprecipitation experiments (not shown).

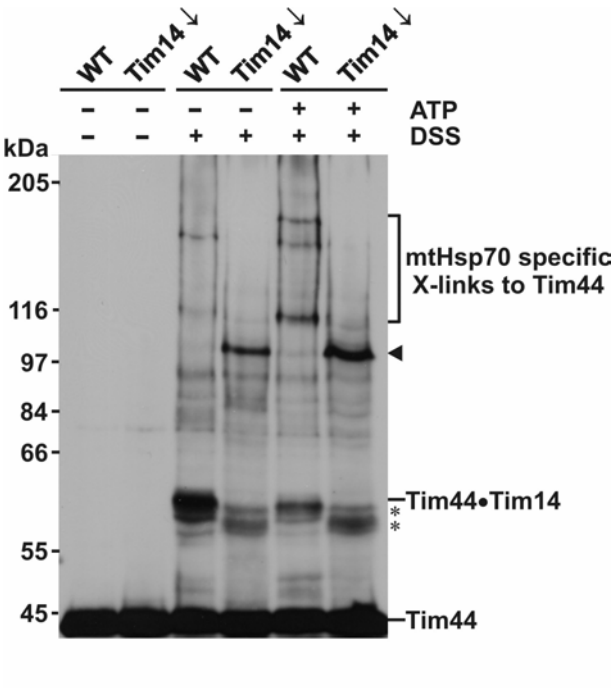

Figure 30. Tim14 interacts with Tim44 in an ATP-dependent manner

Mitochondria from wild type and Tim14 depleted cells were subjected to crosslinking with DSS in the presence of low or of high matrix ATP. Samples were analyzed by SDS-PAGE and immunodecoration with antibodies against Tim44. Crosslinking adducts of Tim44 to unidentified components close in size to Tim14 are indicated by asterisks. Arrowhead indicates a crosslinking adduct of Tim44 which is seen

In conclusion, interaction of Tim14 with Tim44 is modulated by ATP levels in the matrix. Furthermore, Tim14 has also a strong influence on other interactions of Tim44.

Does Tim14 also interact with mtHsp70? To test this, wild type mitochondria were subjected to crosslinking with DSG and samples analyzed by SDS-PAGE and immunodecoration with Tim14 antibodies (Figure 31, left panel). Two strong crosslinking adducts of ca. 93 kDa and ca. 110 kDa, which according to the size could represent crosslinks to mtHsp70, were observed. They were indeed identified as Tim14-mtHsp70

adducts when the same experiment was repeated with mitochondria harboring His-tagged mtHsp70 (Figure 31, right panel). These two crosslinks could specifically be retained on the NiNTA-agarose beads.

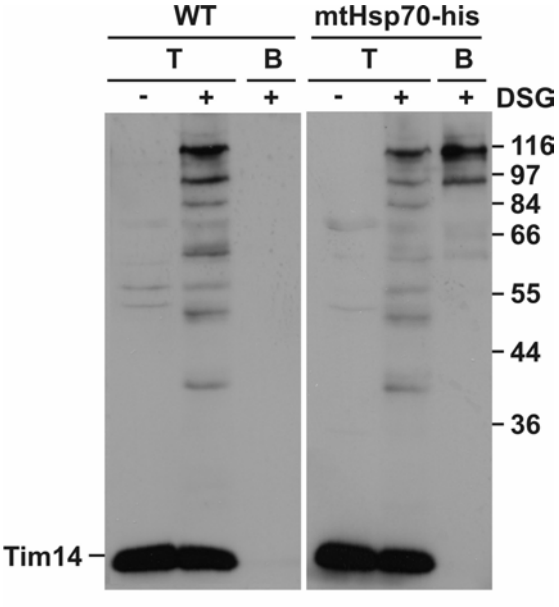

Figure 31. Tim14 can be crosslinked to mtHsp70

Tim14 neighborhood was analyzed by crosslinking in mitochondria isolated from wild type or from cells carrying a His-tag at the C terminus of mtHsp70. Crosslinking adducts were either directly analyzed by SDS-PAGE or were first bound to the NiNTA-agarose beads and, after elution, analyzed by SDS-PAGE and immunodecoration with Tim14 antibodies. T, total crosslinks; B, NiNTA-agarose bound crosslinks.

ATP-dependence of this interaction was then analyzed. Crosslinking was done in wild type mitochondria whose ATP levels were previously manipulated, as described above. Indeed, crosslinking adducts of Tim14 with mtHsp70 could almost exclusively be observed at high ATP levels (Figure 32, leftmost panel). This is again in contrast to the adduct of Tim14 with Tim44 which was stronger in ATP-depleted mitochondria, now analyzed with Tim14 antibodies. Furthermore, the *ssc1-3* mutant strain was included in the analysis. This strain harbors a mutation in the ATPase domain of mtHsp70 and it shows a temperature-sensitive growth phenotype (Gambill *et al.*, 1993). The phenotype can be induced *in organello* by preincubation of mitochondria for 10 min at 37^{o}C. Even at permissive temperature crosslinking adducts of Tim14 to mtHsp70 in *ssc1-3* mitochondria were weaker. After exposure of mitochondria to 37°C, they completely disappeared (Figure 32, rightmost panel), in contrast to the situation in wild type mitochondria. Importantly, a crosslink of Tim14 with Tim44 was still present in *ssc1-3* mitochondria at both permissive and nonpermissive temperatures, showing that mtHsp70 is not required

for Tim14 to interact with Tim44. As expected, however, this crosslink was not ATP-dependent anymore.

In conclusion, Tim14 interacts with mtHsp70 in an ATP-dependent manner. This interaction showed the opposite behavior when compared to the interaction of Tim14 with Tim44. MtHsp70 is not needed for the interaction of Tim14 with Tim44 but in its absence the ATP dependence of this interaction is lost.

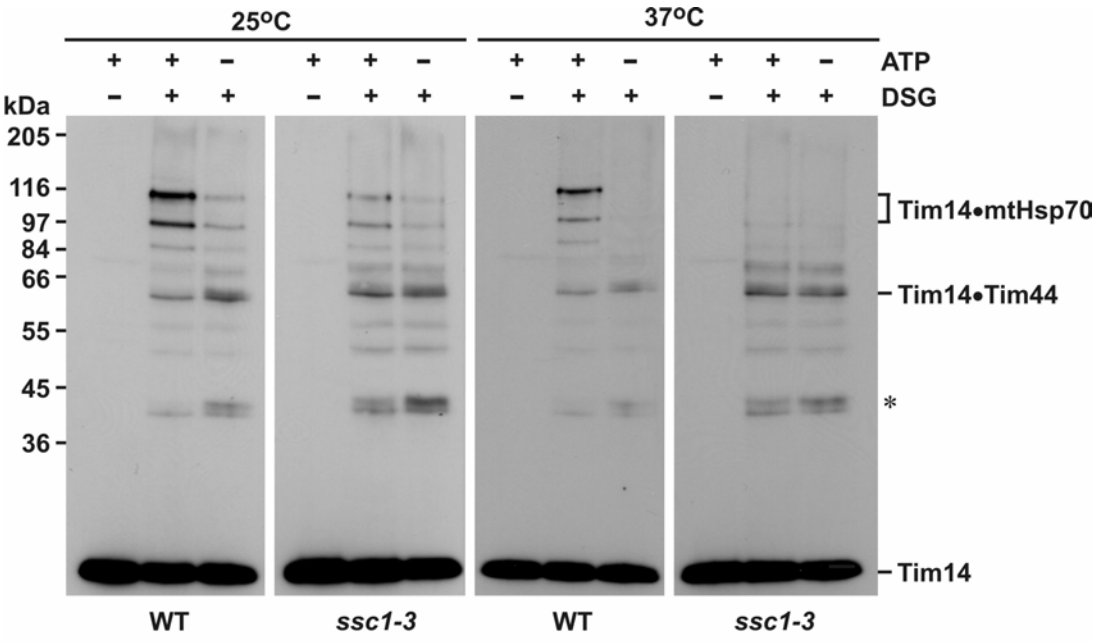

Figure 32. Tim14 crosslinking to mtHsp70 is deficient in the *ssc1-3* mutant at non-permissive temperature

Mitochondria isolated from wild type or from *ssc1-3* mutant cells were preincubated for 10 min at 25°C or at 37°C under conditions that either keep matrix ATP levels high or lead to its depletion. Samples were afterwards subjected to crosslinking with DSG and analyzed by SDS-PAGE and immunodecoration with Tim14 antibodies. Asterisk indicates an unidentified crosslink of Tim14.

To further analyze the role of Tim14 in the import motor of the TIM23 translocase, specific TIM23 substrates which are not dependent on the import motor were analyzed. These precursors have a transmembrane segment directly after the matrix targeting signal (MTS). The MTS is translocated across TIM23 channel in a $\Delta\Psi$-dependent manner and the transmembrane segment is laterally sorted into the inner membrane. If the stretch between MTS and transmembrane segment is short enough, the latter apparently gets so

close to the putative lateral opening of the channel that it can insert without the necessity of the active motor to mediate further translocation. One precursor with such characteristics is pCoxVaΔ(26-89) in which the segment between MTS and the transmembrane anchor was shortened by genetic manipulation. Import of this precursor was shown to require ΔΨ but not the functional motor (Gartner *et al.*, 1995). When the import efficiency of this precursor into wild type and Tim14-depleted mitochondria was compared, almost no difference was observed (Figure 33), in contrast to the full length precursor of pCoxVa (see Figure 27D).

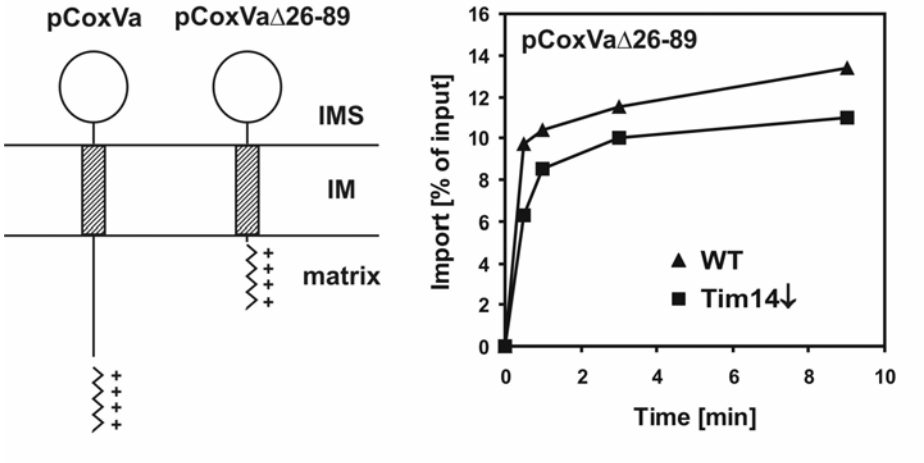

Figure 33. TIM23 substrates which do not need the import motor are not affected by Tim14 depletion

The precursor pCoxVaΔ(26-89) was synthesized in the reticulocyte lysate in the presence of ^{35}S-methionine and imported into wild type and Tim14-depleted mitochondria. Left panel: schematic representation of the topologies of precursors proteins pCoxVa and pCoxVaΔ(26-89). Right panel: quantification of the imported material at various times.

This observation suggests that Tim14 is required for protein import via the TIM23 translocase when its import motor is needed.

Taken together, all these experiments, including the observations that Tim14 needs the intact J-domain for its function (Mokranjac *et al.*, 2003b), strongly support the hypothesis that the primary function of Tim14 is the stimulation of the ATPase activity of mtHsp70 during protein import via TIM23 translocase.

4. DISCUSSION

4.1. TIM23 translocase of *N. crassa*

The biogenesis of mitochondria requires import of several hundred proteins from the cytosol. The TIM23 translocase is the main entry point for mitochondrial matrix and inner membrane proteins. It consists of the inner membrane embedded translocation channel and the import motor which is associated at the matrix side. A lot of information has been obtained in the past years about the role of the TIM23 complex in import. Still, it is highly desirable for a more detailed structural and biochemical analysis to have the complex available in the chemically pure form. In particular, it is important to have the complete set of the TIM23 components identified. This study describes the first purification and initial characterization of the TIM23 complex from *N. crassa*. The *tim23* gene of *N. crassa* was identified using a PCR approach with degenerated primers which were designed based on the homology among known fungal Tim23 proteins. Similar approaches were used to identify the *N. crassa* genes for the other two known components of the TIM23 translocase, *tim17* and *tim44* (F. Nargang and C. Kozany, unpublished results). Subcellular localization experiments with *N. crassa* Tim proteins revealed that indeed all three of them are exclusively found in mitochondria. Like their homologues in other organisms, *N. crassa* Tim17 and Tim23 are integral membrane proteins of the inner membrane (Dekker *et al.*, 1993; Emtage and Jensen, 1993; Maarse *et al.*, 1994; Ryan *et al.*, 1994). It was observed that the N terminus of yeast Tim23 is tethered to the outer membrane and is partially accessible to an externally added protease in intact mitochondria (Donzeau *et al.*, 2000). Experiments with *N. crassa* mitochondria did not reveal a two-membrane spanning topology of Tim23 in this organism. Tim23, like Tim17, was accessible to the externally added protease only after opening of the outer membrane. It remains possible that the *N. crassa* Tim23 also crosses the outer membrane but that the exposed residues are not accessible to the proteases tested. In addition, the N terminus could interact with but not span the outer membrane. In agreement with the behavior of yeast, rat or human Tim44 (Bauer *et al.*, 1999a; Ishihara and Mihara, 1998), *N. crassa* Tim44 was accessible to the externally added protease only after opening of the inner membrane and was completely extractable with carbonate, showing its localization in the mitochondrial matrix. Upon salt extractions of mitochondrial membranes, *N. crassa*

Tim44 was found in two pools – one soluble in the matrix and one stably bound to the membrane, even in the presence of 1.5 M salt. In this respect, *N. crassa* Tim44 resembles more its homologues from higher eukaryotes then the one from yeast. This is also the case for its interaction with Tim17-Tim23 subcomplex. Upon solubilization of mitochondria, *N. crassa* Tim44, like human and rat Tim44, was not found associated with Tim17-Tim23 subcomplex. Experiments performed with human and rat mitochondria left the possibility that this was due to the experimental conditions used, but this study presents clear evidence that in *N. crassa* Tim44 is not stably associated with the Tim17-Tim23 subcomplex either under low or high salt conditions. No interaction was detected in co-immunoprecipitation experiments or in His-tag "pull-down" experiments. However, Tim44, like the other import components Tim17 and Tim23, interacts with the translocating chain and is hence involved in the import into mitochondria. It is therefore reasonable to assume that the interaction of Tim44 with the membrane embedded part of the translocase does exist *in vivo* and is just destroyed upon solubilization of mitochondria. Whether it is mediated by one of the known components of the TIM23 complex or by a so far unidentified one remains to be shown. In any case, the observation that the pool of membrane bound Tim44 could not be released from the membrane by a high salt treatment argues for the involvement of hydrophobic interactions. Even though it is difficult to distinguish whether two pools of Tim44 are in constant exchange or not, their mere existence would suggest a dynamic interaction of Tim44 with the translocation channel.

Quantification of Tim17 and Tim23 within mitochondria demonstrated that they are present in roughly equal molar amounts. These two proteins form a complex which could be detected both by co-immunoprecipitation and "pull-down" experiments with His-tag forms of the proteins. Co-immunoprecipitation experiments demonstrated that antibodies to Tim17 deplete both Tim17 and Tim23 from the detergent extracts. The same results were obtained when antibodies to Tim23 were used for precipitation and when mitochondria were solubilized in the presence of high or low concentrations of salt. These experiments demonstrate that there is no free pool of either protein present in mitochondria. When "pull-down" experiments were done from detergent extracts of mitochondria containing both wild type and His-tagged Tim23, the wild type version could not be co-isolated with the tagged one. As the tagged version is fully functional *in vivo* it is unlikely that that the tag is interfering with the oligomerization of Tim23. It is, however, possible that the dimers of Tim23 are destroyed upon solubilization, even under

very mild conditions. Taken together, these experiments show that Tim17 and Tim23 form a stable subcomplex which probably contains one copy of each protein.

Small-scale purification trials were made in order to establish the optimal purification procedure. The optimization procedure included a) choice of the position of the His-tag, b) choice of the detergent which keeps Tim17 and Tim23 together, c) choice of the starting material (whole mitochondria or purified membranes) and d) choice and optimization of the two chromatography procedures. In the first step, purification of the complex was tried from strains expressing Tim23 with either N- or C-terminal His-tag. Both tags enabled purification of Tim17 along with the tagged Tim23. However, the N-terminal tag was chosen for further optimizations as it was binding considerably better to the NiNTA-agarose beads. This is probably due to a better accessibility of the N-terminal tag which is farther away from the membrane embedded part of Tim23. In the next step, stability of the TIM23 complex was tested in a whole series of detergents. Digitonin turned out to be the only one which did not destroy Tim17-Tim23 interaction. The necessity for the mildest detergent available shows the very labile nature of the *N. crassa* TIM23 complex. For comparison, the TOM core complex is stable in dodecyl-maltoside and use of digitonin is necessary only to keep both receptors bound to the complex. The TIM23 complex was finally purified from mitochondrial membranes obtained from a strain expressing an N-terminally tagged Tim23. They were solubilized with digitonin and the complex purified by two chromatography steps, NiNTA-agarose affinity chromatography followed by an anion exchange chromatography. Purification steps were monitored by SDS-PAGE and immunoblotting for Tim17 and Tim23 combined with staining for total proteins by silver or with the Comassie Brilliant Blue. As was expected from small scale experiments, Tim44 did not co-purify with the membrane embedded part of the translocase. However both kinds of staining revealed that one protein did co-purify with Tim17 and Tim23 after both chromatographic steps and therefore possibly represented a new Tim protein (see below).

Availability of the purified complex should enable a more detailed structural and biochemical analysis in the future. Image analysis by electron microscopy would give first insights into the structure. In particular, reconstitution into lipid vesicles and confirmation of the translocation activity would be desirable. However, it has to be kept in mind that this complex lacks the import motor part of the translocase which is essential for the complete translocation of matrix targeted precursors across the inner membrane. In order to avoid the need for the import motor, a specific subset of precursors might be used. In

particular, TIM23 precursors which are laterally sorted into the inner membrane can be imported in the absence of functional mtHsp70. Their dependence on Tim44 has not been checked (Gärtner *et al.*, 1995; Rojo *et al.*, 1998). Provided that these precursors do not need Tim44 for their import, purified *N. crassa* TIM23 complex reconstituted into lipid vesicles may provide valuable insights into the lateral opening of the translocation channel, a process which, so far, is entirely unknown.

4.2. Essential role of Tim50 in transfer of preproteins from TOM to TIM23 complex

Mass spectrometric analysis of the protein co-purifying with *N. crassa* TIM23 complex revealed a protein of a so far unknown function whose homologues are present in virtually all eukaryotes. Based on the established nomenclature it was named Tim50 (Pfanner *et al.*, 1996). Subcellular and submitochondrial localization experiments demonstrated that *N. crassa* Tim50 is found exclusively in mitochondria. There it is anchored in the inner membrane by a single transmembrane domain and exposes a large C-terminal domain into the IMS. All found homologues have the same predicted topology – an N-terminal matrix targeting signal, followed by a transmembrane domain and a large C-terminal domain. Clear sequence homology is, however, restricted to the C-terminal, IMS exposed domain. Tim50 is specifically associated with the TIM23 translocase in *N. crassa* as demonstrated both by co-immunoprecipitation and His-tag pull-down experiments. It is rather clear that Tim50 interacts with the translocation channel via Tim23 as these two proteins were found in one complex in the absence of Tim17. This is in the agreement with the observations made by two other groups who independently identified Tim50 in yeast and who showed that the N-terminal domain of Tim23 and the C-terminal domain of Tim50 interact in a two-hybrid assay (Geissler *et al.*, 2002; Yamamoto *et al.*, 2002).

Tim50 is essential for viability for both yeast and *N. crassa*, in agreement with the essential nature of all other TIM23 components known ((Mokranjac *et al.*, 2003a) and S. Hoppins and F. Nargang, unpublished results). Furthermore, mitochondria isolated from yeast cells depleted of Tim50 showed severe import defects of TIM23 substrates both *in vivo* and *in vitro* (Mokranjac *et al.*, 2003a). This is a specific defect of the TIM23 translocase as depletion of Tim50 did not impair the function of either TOM or TIM22 complex. Its role in the translocation process is further supported by the fact that Tim50 interacts with, or at least is in the close proximity of the translocating precursors which

use the TIM23 translocase. In summary, these results show that Tim50 has a distinct role in the TIM23 import pathway. Remarkably, Tim50 appears to be the first component of the TIM23 translocase which gets in contact with precursors during import as cross-linked adducts of Tim50 were already observed to precursors only partially translocated across the outer membrane. Tim50 is also in close neighborhood of precursors which are partially translocated across the inner membrane suggesting that it interacts with various segments of the precursor as they are entering and crossing the IMS. Interestingly, both co-immunoprecipitation and His-tag pull-down experiments demonstrated that only about 10-20% of total Tim50 was associated with the TIM23 translocase. Even though it is possible that the interaction is labile and partially destroyed upon solubilization, these findings raise the possibility that Tim50 interacts in a dynamic fashion with the TIM23 translocation channel. Taken together, all these experiments support the hypothesis that the main function of Tim50 is to bind MTS-containing precursors as they appear from the TOM complex and transfer them to the translocation channel of the TIM23 complex (Figure 34).

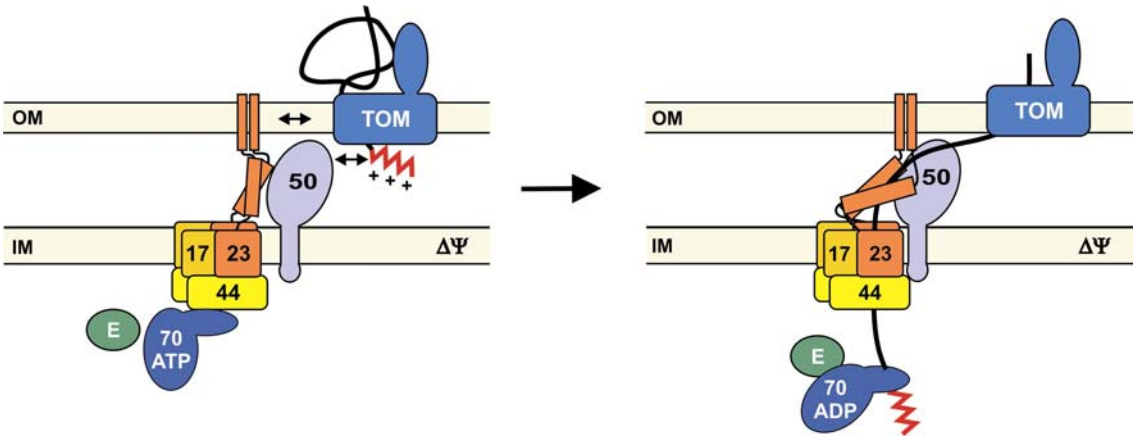

Figure 34. Model of Tim50 function

Experimental evidence supports the hypothesis that the major function of Tim50 is to transfer precursors from TOM to TIM23 complex. Presequence-containing precursors, only partially translocated across the outer membrane, are already bound to Tim50. Tim50 thus appears to be the first component of the TIM23 complex which interacts with the translocating chain as soon as it appears at the outlet of the TOM complex. Tim50 then guides the precursor protein to the translocation channel of the TIM23 complex which translocates it across the inner membrane.

Future studies will have to address this hypothesis in more detail. For example, it would be interesting to see if mitoplasts prepared from Tim50-depleted mitochondria still display import defects. If the major function of Tim50 is to transfer precursors from the TOM to the TIM23 complex one may expect that in mitoplasts, where precursors have a direct access to the translocase in the inner membrane, Tim50 may not be needed. Furthermore, it would be also interesting to see if any interaction between Tim50 and the TOM complex could be detected. Indeed, some preliminary data show that Tim50 in intact mitochondria can be cross-linked to several proteins whose identification awaits further analysis (own unpublished observations). It is tempting to speculate that one of them might be a TOM component.

4.3. Tim14, the J-protein in the mitochondrial protein import motor

The purification of the *N. crassa* TIM23 translocase enabled the identification of Tim50, a so far overlooked essential component of the translocase. On the other hand, Tim44, a known component of the complex, did not co-purify with it. This raised considerable concerns that some components have still remained overlooked. In particular, the presence of an unknown component of about 15-20 kDa which interacts with Tim44 has been implicated in the literature (Moro *et al.*, 1999). To identify this component an *in silico* approach was used. In this respect, yeast is a much better model system compared to *N. crassa* due to the completely sequenced genome and the availability of the several genome-wide analyses of deletion mutants and subcellular localization of encoded proteins. Yeast databases were searched for a conserved, essential protein of unknown function with a molecular mass between 15 and 20 kDa and a mitochondrial targeting signal. Protein encoded by the *YLR008c* reading frame fulfilled all the above criteria and was therefore tested if it was the right one. Cross-linking experiments demonstrated that indeed the right protein was identified and was named Tim14. The predicted molecular mass of the protein encoded by *YLR008c* is 17.9 kDa but assigning the affix 18 to this Tim, according to the established nomenclature (Pfanner *et al.*, 1996), was not possible as Tim18 already exists as well as Tim17. Since all other putative homologues have lower molecular masses, between 11 and 16 kDa, the affix 14 was chosen.

The identification of Tim14 considerably advances the understanding of the function and structural organization of the TIM23 translocase. Tim14 is exclusively found in mitochondria where it is anchored in the inner mitochondrial membrane by a single

transmembrane domain and exposes a conserved C-terminal domain into the matrix. Tim14 not only cross-links to Tim44 but it is an integral component of the TIM23 complex. Co-immunoprecipitation experiments demonstrated that a considerable fraction of Tim14 associates with the TIM23 translocase. Furthermore, they showed that Tim44 is necessary for Tim14 recruitment to the translocase. In the absence of Tim14 the rest of the translocase assembles normally. Still, mitochondria depleted of Tim14 were severely impaired in the import of most TIM23 substrates. In fact, the only TIM23 substrates which were imported at almost wild type rates were the ones whose import was shown not to depend on the functional mtHsp70. These results would suggest a specific role of Tim14 in the import motor part of the translocase. Such a role of Tim14 was further supported by ATP-dependent cross-links to both Tim44 and mtHsp70. The additional evidence for the involvement in the import motor comes from the sequence of Tim14 itself. Tim14 exposes a classical J-domain into the matrix. According to the sequence similarity and structural predictions, this J-domain contains a canonical HPD motif and typical helical segments H1-H3 found to make up a tertiary fold of J-domains (Qian *et al.*, 1996; Szyperski *et al.*, 1994). The function of all known J-domain proteins is to stimulate the ATPase activity of Hsp70 chaperones (Kelley, 1998). Indeed, a mutant form of Tim14 in which the absolutely conserved histidine in HPD motif is changed to glutamine does not support growth of yeast cells (Mokranjac *et al.*, 2003b). Taken together, all these data strongly support the hypothesis that Tim14 is needed for the stimulation of the ATPase activity of mtHsp70 during protein translocation into the matrix.

The evolutionary conservation between the *E. coli* DnaK-DnaJ-GrpE folding machine and the mitochondrial protein import motor seems much more evident in the light of the results obtained in this study. In the bacterial system, DnaJ consists of the N-terminal J-domain, which binds to the ATPase domain of DnaK and stimulates its ATPase activity, and the more C-terminally located peptide binding segment (Banecki *et al.*, 1996; Liberek *et al.*, 1991; McCarty *et al.*, 1995; Szabo *et al.*, 1996; Wall *et al.*, 1994). In the mitochondrial system, Tim14 contains a J-domain while Tim44 has the ability to bind preproteins (Blom *et al.*, 1993; Rassow *et al.*, 1994; Schneider *et al.*, 1994). In this respect, Tim14 and Tim44 can be viewed as two parts of DnaJ. Functional conservation may go even further. The peptide binding domain of DnaK was reported to be able to interact with DnaJ (Suh *et al.*, 1998; Suh *et al.*, 1999). In mitochondria, the peptide binding domain of mtHsp70 was reported to interact with Tim44 (Moro *et al.*, 2002), although a different view has been put forward (Krimmer *et al.*, 2000).

Based on results obtained the following model for the function of the import motor can be proposed. Tim44 recruits mtHsp70 in its ATP form to the translocation channel. The unfolded preprotein appearing at the outlet of the channel binds to the peptide binding domain of mtHsp70. Tim14 stimulates the ATPase activity of mtHsp70 and, as a result, the peptide binding pocket closes and the preprotein is tightly bound. The mtHsp70 dissociates from the membrane and the preprotein bound to mtHsp70 can move into the matrix, but not in a retrograde fashion. The mitochondrial nucleotide exchange factor Mge1 mediates the release of ADP from mtHsp70 followed by the release of the preprotein so that mtHsp70 can be engaged in the new cycle of the motor. The activation of mtHsp70 by Tim14 is an essential element of the cycle as the ATP hydrolysis is the rate-limiting step in the cycles of all known Hsp70 chaperones (Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002). In the absence of Tim14, mtHsp70 can not bind tightly the preprotein and therefore it can slide back into the translocation channel.

The mitochondrial protein import motor shows clear resemblance to the bacterial DnaK-DnaJ-GrpE system, as discussed above, but it also shows one marked difference. Namely, Tim14, Tim44 and mtHsp70 are assembled at the site of action, the translocation pore of TIM23 complex, even without the translocating chain and this raises a question to the model proposed above for the function of the import motor. How is the ATP/ADP cycle of mtHsp70 controlled so that mtHsp70 is not in an idling state when no polypeptide substrate is presented by Tim44? It is possible that the stimulation of mtHsp70 by Tim14 is controlled by another component that senses the presence or absence of the translocating chain. Indeed, Tim14 is found in a stable complex with Tim16, the most recently identified component of the import motor. According to the structure prediction programs, Tim16 contains three typical helices found to make up the tertiary fold of the J-domain but lacks the canonical HPD motif (C. Kozany *et al.*, Nature Structural and Molecular Biology, in press). It is tempting to speculate that Tim16 regulates the activation of mtHsp70 as a nonfunctional J-protein by binding to mtHsp70 in the absence of the translocating precursor. In the presence of the translocating chain, Tim16 would dissociate and the import motor would rearrange so that Tim14 can interact with and stimulate the ATPase activity of mtHsp70.

Further points to clarify include the exact sequence of events following binding of the translocating precursor to mtHsp70. Whether ATP hydrolysis precedes the dissociation of mtHsp70 from Tim44 or mtHsp70 is released from Tim44 in the ATP form and Tim14 stimulates the ATP hydrolysis afterwards is currently unknown. Availability of the

purified components should enable questions like this one to be tackled (Liu *et al.*, 2003). It is, however, essential that all components are known as a missing one could lead to misconceptions. Furthermore, it is difficult to predict in which way the membrane embedded part of the translocase influences the import motor and it is possible that the reaction cycle of the motor in the context of the membrane is different.

Another interesting point is the fact that yeast mitochondria contain Mdj2, a protein which is 55% identical to Tim14 but whose deletion has no obvious phenotype. The alignment of Mdj2 with Tim14 and their homologues from *C. elegans* and *H. sapiens* reveals several regions which are conserved among putative Tim14 homologues but not in Mdj2 (Figure 35). It will be interesting to see if these residues are essential for binding to Tim44, Tim16 or mtHsp70. In this way it could be explained why Tim14, but not Mdj2, is the J-domain protein of the mitochondrial import motor.

Figure 35. Alignment of Tim14 and its homologues from *C. elegans* and *H. sapiens* with Mdj2

Yeast mitochondria contain Mdj2, a protein which is 55% identical to Tim14 but whose deletion has no obvious phenotype. The alignment of Tim14 with its putative homologues and Mdj2 shows that some amino acid residues (circled in red) are conserved between Tim14 and its homologues but not in Mdj2. They are possibly involved in binding to Tim44, mtHsp70 or Tim16.

5. SUMMARY

Mitochondria import the vast majority of their proteins from the cytosol. Correct targeting and sorting within mitochondria is achieved through the concerted action of the translocases present in the mitochondrial outer and inner membranes. The TIM23 complex (translocase of the inner membrane) mediates the membrane potential- and ATP-dependent import of presequence-containing proteins into mitochondria. It is the main entry gate for proteins of the matrix and the inner membrane. The TIM23 complex consists of the membrane embedded translocation channel and of the import motor which is associated with the channel at the matrix side. This study provides new information on the composition and the function of this complex.

In order to analyze the function and the structure of the TIM23 translocase, the complex was purified from *Neurospora crassa* mitochondria. *N. crassa* genes encoding the three known components of the translocase, Tim17, Tim23 and Tim44, were identified by a PCR approach based on the homology among known fungal Tim proteins. Like their homologues from other organisms, *N. crassa* Tim17 and Tim23 are integral inner membrane proteins. *N. crassa* Tim44 is present in two distinct populations – one soluble in the matrix and one stably attached to the inner membrane. All three proteins interact with the translocating precursor and are thus indeed involved in the protein import into mitochondria. *N. crassa* TIM23 complex is highly unstable upon solubilization. Use of digitonin is necessary to keep Tim17 and Tim23 together. Tim44 is not associated with this subcomplex, neither under low nor high salt conditions. An *N. crassa* strain expressing Tim23 with an N-terminal His-tag enabled purification of the TIM23 complex. Besides the known components Tim17 and Tim23, the purified complex contains a novel component named Tim50.

Tim50 is integrated in the inner mitochondrial membrane by a single transmembrane domain and exposes a large C-terminal domain into the IMS. It is encoded by an essential gene both in *N. crassa* and in yeast. Homologues are present in virtually all eukaryotes. Yeast mitochondria depleted of Tim50 displayed severe import defects of preproteins which use the TIM23 translocase. Tim50 is in contact with the translocating preprotein as soon as it appears at the outlet of the TOM complex and as long as its segments are present in the IMS. Thus Tim50 has a major role in the intermembrane space in transferring of preproteins from the TOM complex to the TIM23 complex.

Using a combination of crosslinking experiments and *in silico* analysis, an additional component of the TIM23 translocase was identified and termed Tim14. Tim14 is integrated in the mitochondrial inner membrane and exposes a typical J-domain into the matrix. In yeast, Tim14 is essential for cell viability and its homologues are present throughout the eukaryotic kingdom. Mitochondria depleted of Tim14 are specifically defective in the import of preproteins which use the TIM23 translocase. In particular, import of preproteins is affected which depend on the import motor. Tim14 interacts with Tim44 and mtHsp70 in an ATP-dependent manner. Taken together, these results suggest that Tim14 is a constituent of the import motor where its function is to stimulate the ATPase activity of mtHsp70. In this way this chaperone can act in a rapid and regulated manner in the Tim44-mediated trapping of preproteins entering the matrix.

6. LITERATURE

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7. ABBREVIATIONS

AAC	ADP/ATP carrier
ADP	adenosine diphosphate
Amp	ampicillin
APS	ammonium peroxodisulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BN-PAGE	blue native polyacrylamide gel electrophoresis
BSA	bovine serum albumin
C-	carboxy-
CBB	coomassie brilliant blue
CCCP	carbonyl cyanide m-chlorphenylhydrazone
CCHL	cytochrome *c* heme lyase
cDNA	complementary DNA
CNBr	cyanogen bromide
CSPD	3-(4-methoxyspiro{1,2-dioxetan-3,2´-(5´chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenylphosphate, di sodium salt
CV	column volume
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DHFR	dihydrofolate reductase
DIG	digoxigenin
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DSG	disuccinimidyl glutarate
DSS	disuccinimidyl suberate
DTT	dithiotreitol
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
gDNA	genomic DNA
GIP	general import pore
HA	Haemagglutinin
HEPES	N-2 hydroxyl piperazine-N´-2-ethane sulphonic acid
Hsp	heat shock protein
IgG	immunglobulin G
IM	inner membrane
Imp	inner membrane peptidase
IMS	intermembrane space
IPTG	isopropyl-β,D-thiogalactopyranoside
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
LB	Luria Bertani
m^{7}G(5`)ppp(5`)G	7-methylguanosine triphospate
MBP	maltose binding protein
MOPS	N-morpholinopropane sulphonic acid

MPP	mitochondrial processing peptidase
MSF	mitochondrial import stimulation factor
MTS	matrix targeting signal
MTX	methotrexate
N-	amino-
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
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PAS	protein A-Sepharose
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	preimmune serum
PK	proteinase K
PMSF	phenylmethylsulfonyfluoride
RIP	repeat-induced-point mutation
RNA	ribonucleic acid
RNasin	ribonuclease inhibitor
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
TBS	TRIS buffered 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
v/v	volume per volume
w/v	weight per volume
WT	wild type
$\Delta\Psi$	membrane potential

Publications resulting from this thesis

Steinmetz, LM., Scharfe, C., Deutschbauer, AM., Mokranjac, D., Herman, ZS., Jones, T., Chu, AM., Giaever, G,, Prokisch, H., Oefner, PJ., and Davis, RW. (2002) Systematic screen for human disease genes in yeast. *Nat. Genet.*, **31**, 400-404. Epub 2002 Jul 22.

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