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# MCC and PSC, the Putative Protein Import Channels of Mitochondria[1]

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All but a small fraction of the hundreds of proteins in a mitochondrion are synthesized in the cytoplasm and imported into the organelle. Water-filled channels are integral to the process of translocating proteins since channels can provide an aqueous pathway through the hydrophobic environment of the membrane. The MCC (multiple conductance channel) and PSC (peptide-sensitive channel) are two high-conductance channels previously identified in electrophysiological studies of mitochondrial membranes. MCC and PSC are the putative pores of the import complexes of the inner and outer membranes, respectively. The genetic, biochemical, and biophysical evidence regarding these assignments are summarized herein. These findings support the identification of MCC and PSC as the protein import channels of mitochondria.

**KEY WORDS:** Protein import; mitochondria; MCC; PSC; import pore; protein-translocating channels.

## INTRODUCTION

Protein translocation across membrane barriers is important in many cellular functions including signaling, secretion, biogenesis of organelles, compartmentation, and programmed cell death. Since about one-half of the proteins synthesized in a cell must cross at least one membrane before reaching their final destinations, protein translocation across membranes is a fundamental cellular process (Schatz and Dobberstein, 1996). This process is especially vital for mitochondrial biogenesis since >95% of all mitochondrial proteins must be imported.

Although translocation processes occur across membranes as diverse as the plasma membrane, endoplasmic reticulum, and mitochondrial membranes, certain basic principles seem to apply (Schatz and Dobberstein, 1996). For example, Blobel and Dobberstein (1975) proposed that water-filled pores, or channels, play an integral role in the protein translocation processes and provide a pathway through which proteins may cross membranes. Evidence supporting this idea was provided by the studies of Simon and Blobel (1991, 1992) in which electrophysiological techniques were used to identify potential protein–translocation channels in the endoplasmic reticulum and bacterial cell membranes. In the general protein translocation scheme, unfolded polypeptides are thought to move through membrane channels that open upon binding of targeting signals to receptors. Typically, the targeting regions, or presequences, are located at the amino-terminal end of the precursors and are cleaved after translocation. A molecular motor is thought to "pull" the precursors through the channels and then chaperones catalyze folding of the proteins (Schatz and Dobberstein, 1996).

The general protein translocation scheme can be applied to mitochondrial membranes. Most mitochondrial proteins are synthesized in the cytosol as precursors and carry presequences at their amino-termini.

[1] Key to abbreviations: AAA, alkaline pH-activated anion channel; ACA, alkaline pH-activated cation channel; ANT, adenine nucleotide translocator; IMS, intermembrane space; MCC, multiple conductance channel; mCS, mitochondrial centum picosiemen channel; PSC, peptide-sensitive channel; Tim, translocase of inner membrane; Tom, translocase of outer membrane; VDAC, voltage-dependent anion-selective channel.

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These presequences contain the information that targets proteins to mitochondria, they are removed during or after import into the organelles (Roise and Schatz, 1988; Lemire *et al.*, 1989; Allison and Schatz, 1986). While there is little or no sequence homology among mitochondrial presequences, they typically carry a net positive charge and assume an amphipathic α-helical structure. Synthetic peptides, whose sequences mimic these regions, are referred to as "targeting peptides" and they competitively inhibit protein import into mitochondria (Glaser and Cumsky, 1990; Hugosson *et al.*, 1994). This inhibition is thought to reflect a competition between mitochondrial precursors and targeting peptides for binding sites on receptors on the outer membrane. Most precursors are translocated across one or both of the mitochondrial membranes, at least in part, because of the interaction of their targeting regions with the protein import machinery.

Import of precursors into mitochondria is a multistep process involving many proteins. These proteins are organized into complexes, called Tim (translocase of the inner membrane) and Tom (translocase of the outer membrane), that catalyze import across the two membranes (Pfanner *et al.*, 1996)(Fig. 1). Both Tim and Tom complexes have receptors necessary for recognition and sorting of precursors as well as a pore for selective translocation across the membranes. Tim and Tom designate components of complexes followed by their molecular weight. As shown in the model of Fig. 1, the MCC (multiple conductance channel) (Lohret *et al.*, 1997; Jensen and Kinnally, 1997; Kinnally *et al.*, 1996; Lohret and Kinnally, 1995) and PSC (peptide-sensitive channel) (Kunkele *et al.*, 1998; Chich *et al.*, 1991; Fevre *et al.*, 1990, 1994; Henry *et al.*, 1989; Juin *et al.*, 1997) are the putative import pores of the Tim and Tom complexes, respectively. The evidence supporting this assignment and more comprehensive descriptions of the Tim and Tom complexes are detailed below.

## THE TOM COMPLEX

The outer membrane is a physical barrier representing the first level of selection for precursor proteins synthesized in the cytoplasm, regardless of their final destination within the mitochondrion. The Tom complex is responsible for recognition of the mitochondrial presequences and for translocation of precursors across the outer membrane (Fig. 1). Importantly, protein translocation was recently reconstituted in proteoliposomes containing purified Tom complex in the elegant study of Kunkele *et al.* (1998). Furthermore, incorporation of the purified Tom complex in planar bilayers imparted a channel activity that was modified by synthetic targeting peptides. These findings clearly demonstrate the association of channel activity with a protein complex capable of translocating proteins across membranes. In addition, single-particle electron microscopic image analysis showed the presence of two, and possibly three, apparent pores associated with each purified Tom complex.

The purified and reconstituted Tom complex was functional and contained six proteins (Kunkele *et al.*, 1998). These components were Tom40p, Tom22p, Tom20p, Tom70p, Tom7p, and Tom6p. Tom22p and Tom20p form a receptor subcomplex that recognizes the general mitochondrial presequence, which is cationic and α-helical (Lithgow and Schatz, 1995). Another receptor subcomplex is formed by the association of Tom70p with Tom37p and recognizes the signal-denoting membrane proteins (Lithgow *et al.*, 1994). Tom6p and Tom7p modulate the association of both subcomplexes with Tom40p (Alconada *et al.*, 1995). While Tom22p is an essential protein, knockout mutants of Tom20p and Tom70p are viable (Lithgow *et al.*, 1994). Interestingly, none of the Tom proteins have been assigned a "motor" activity for "pulling" the precursor through the pore of the complex.

The essential protein Tom40p was identified as the import pore of the Tom complex. In another recent study, Hill *et al.* (1998) found that bacterially expressed Tom40p imparted channel activity to planar bilayers. Targeting peptides modified the channel activity associated with Tom40p, like that of the Tom complex (Kunkele *et al.*, 1998).

## THE TIM COMPLEX

The Tim complex, or translocase of the inner membrane, catalyzes the import of proteins across the inner membrane (see Fig. 1). Typically, several steps are needed to import precursors, including binding to outer membrane receptors and translocation across one or both mitochondrial membranes. Import through the Tim complex can operate independently of the Tom complex since import occurs in mitoplasts (mitochondria stripped of their outer membranes) (Emtage and Jensen, 1993, Lithgow *et al.*, 1994). However, current models favor their transient linkage at contact sites (junctions where the two membranes are closely apposed) (Hackenbrock, 1968; Lithgow *et al.*, 1995;

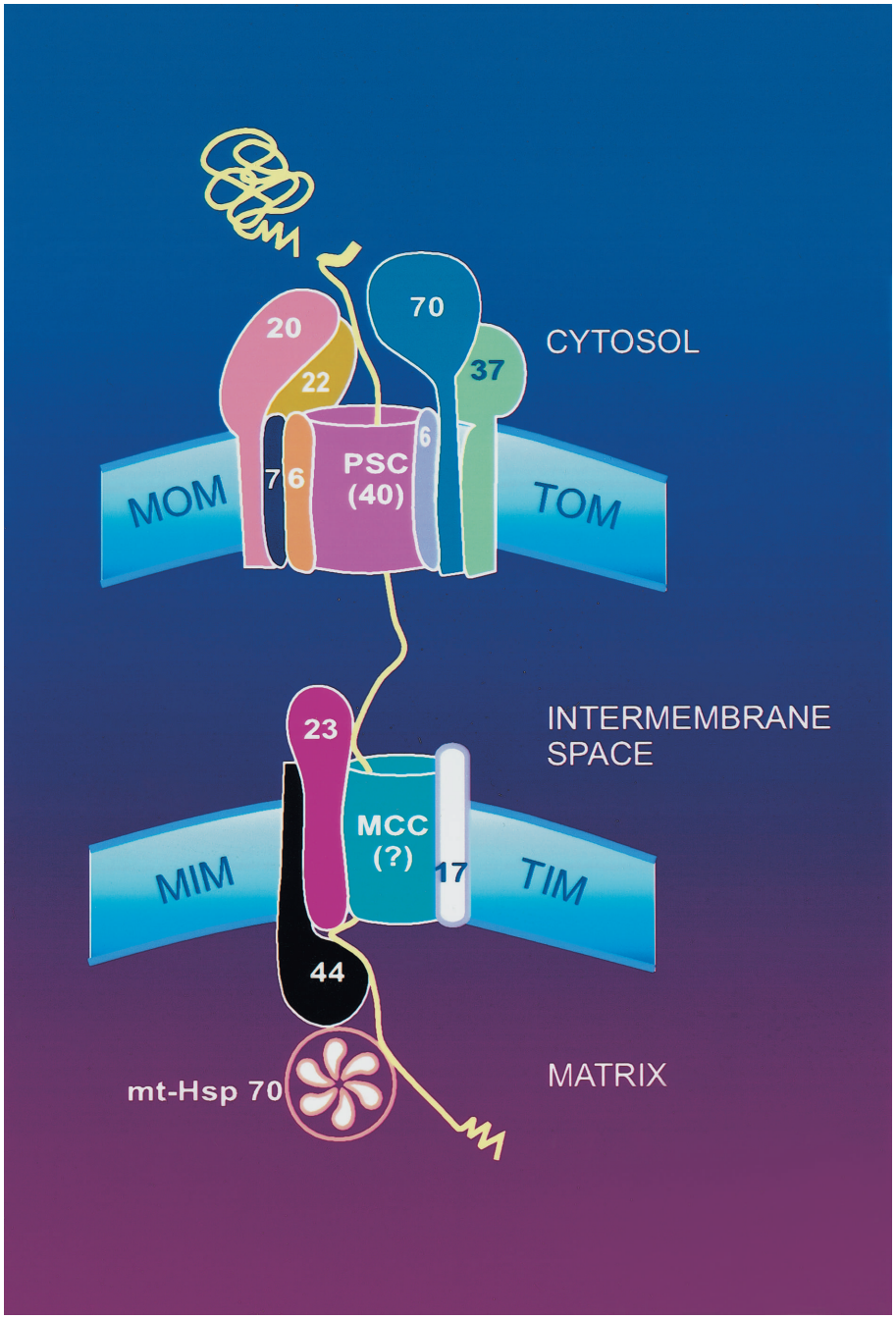


**Fig. 1.** The protein import machinery of mitochondria. Multisubunit complexes called Tim and Tom are located in the mitochondrial inner (MIM) and outer (MOM) membranes. MCC and PSC form channels in these complexes that allow the passage of precursors (yellow) from the cytosol to the matrix.

Glick *et al.*, 1991). In an elegant, electron-microscopic study by Schulke *et al.*, (1997), the inner and outer membranes were ™zipperedſ together by stalling precursors in the import machinery.

Although characterization of the Tim complex is not as complete as that of the Tom complex, some differences have emerged. Translocation through the Tom complex requires ATP. However, translocation of

precursors across the inner membrane requires ATP and an electrochemical potential, which is normally established by the electron transport chain (Gasser *et al.*, 1982; Martin *et al.*, 1991; Schleyer *et al.*, 1982). The membrane potential is thought to play a role in translocation of the presequence across the inner membrane. While none is apparently associated with the Tom complex, the Tim complex has a "motor" to facilitate transport of the precursors through the pore. A member of the hsp70 family (mt-hsp70p) associates with Tim44p to form another subcomplex on the matrix face of the inner membrane (Kang *et al.*, 1990; Ungermann *et al.*, 1994, 1996; Horst *et al.*, 1993; Scherer *et al.*, 1992; Blom *et al.*, 1993; Maarse *et al.*, 1994). These two proteins form, at least in part, an ATP-driven import motor. The subcomplex binds the precursor and a conformational change in mt-hsp70p (brought on by ATP hydrolysis) generates the torque that "pulls" precursors through the transmembrane pore of the Tim complex (Schneider *et al.*, 1994; Rassow *et al.*, 1994). The mt-Hsp70p, retained by the precursor after its release from Tim44p, prevents the unfolded polypeptide from sliding back through the pore.

While the exact functions of Tim23p and Tim17p in import are not known, it has been suggested that both proteins form part of the channel in the inner membrane through which precursors are translocated into the matrix (Ryan and Jensen, 1993; Emtage and Jensen, 1993; Berthold *et al.*, 1995; Bauer *et al.*, 1995; Dekker *et al.*, 1993; Kubrich *et al.*, 1994). Both Tim23p and Tim17p are essential proteins with several transmembrane domains that are closely associated in the Tim complex (Ryan *et al.*, 1998; Ryan and Jensen, 1993; Emtage and Jensen, 1993; Blom *et al.*, 1993; Maarse *et al.*, 1994; Berthold *et al.*, 1995). Both Tim23p and Tim17p can be chemically cross-linked to a precursor arrested in transit across the inner membrane (Ryan and Jensen, 1993), and depletion of Tim23p from cells results in a defect in import (Emtage and Jensen, 1993). Furthermore, antibodies to Tim23p inhibit import of several mitochondrial proteins across the inner membrane (Emtage and Jensen, 1993). However, as shown below, Tim23p is most likely a receptor and is not required for the basic structure of the pore of the Tim complex.

Recently, three new essential genes were identified as possible components of the Tim complex. Tim11p is associated with precursors that sort to the space between the two membranes (Tokatlidis *et al.*, 1996). Tim22p and Tim54p form a system distinct from the Tim23p–Tim17p-containing complex (Kerscher *et al.*, 1998; Haucke and Schatz, 1997) and are thought to be responsible for insertion of membrane proteins. Tim22p and Tim54p apparently do not require the presence of Tim44p and mt-hsp70 to function (Kerscher *et al.*, 1998). Several laboratories suggest other members of the Tim complex have yet to be identified. For example, immunoprecipitation and cross-linking studies implicate the involvement of proteins with molecular weights of 14, 20, and 33 kDa (Blom *et al.*, 1993; Berthold *et al.*, 1995; Kerscher *et al.*, 1998).

## EXPECTATIONS FOR THE PROTEIN IMPORT PORES OF MITOCHONDRIA

While Blobel and Dobberstein (1975) proposed that water-filled pores or channels played an integral role in protein translocation processes almost 25 years ago, the experimental evidence supporting this idea was only recently obtained. Several predictions can be made for the characteristics of the protein import pores based on the general protein translocation scheme. The pores of the Tim and Tom complexes will have diameters consistent with their ability to allow the selective passage of unfolded polypeptides. Presequences will modulate the opening of the pore of the complex. Structural modifications of the complexes (*e.g.*, by mutation or protease treatment) and/or antibodies against complex components that modify protein import may alter single-channel behavior or modulation by presequences. These criteria are being used to evaluate whether specific channels, previously identified by electrophysiological techniques, are associated with protein translocation in the Tim and Tom complexes of mitochondria.

## MCC AND PSC, TWO MITOCHONDRIAL CHANNELS

MCC (multiple conductance channel) and PSC (peptide-sensitive channel) are channels located in the inner and outer membranes of mitochondria, respectively. While MCC was originally described in patch-clamp studies of native inner membranes (Kinnally *et al.*, 1989), PSC was discovered using the tip-dip method on reconstituted mitochondrial membranes (Henry *et al.*, 1989). In recent studies, inner and outer membranes have been purified and fused with liposomes to form large proteoliposomes. Membrane

patches are excised from the proteoliposomes with a micropipette and the flow of ions, or current, through the channels is examined in the presence or absence of effectors, *e.g.*, targeting peptides or antibodies.

The activities of MCC and PSC are highly conserved among mouse, yeast, and *N. crassa* mitochondria, which is consistent with both channels having roles in fundamental cellular processes (Lohret and Kinnally, 1995; Kunkele *et al.*, 1998; Fevre *et al.*, 1990). As expected from the low permeability of the inner membrane of mitochondria, MCC of mitoplasts is normally closed under metabolizing conditions, but it can be activated, *e.g.*, by μM targeting peptides or μM calcium (Kinnally *et al.*, 1991, 1992, 1996; Kushnareva *et al.*, 1999). In addition, MCC activity is usually detected after its reconstitution into proteoliposomes, suggesting that regulatory components are lost during the fractionation procedure (Lohret *et al.*, 1996).

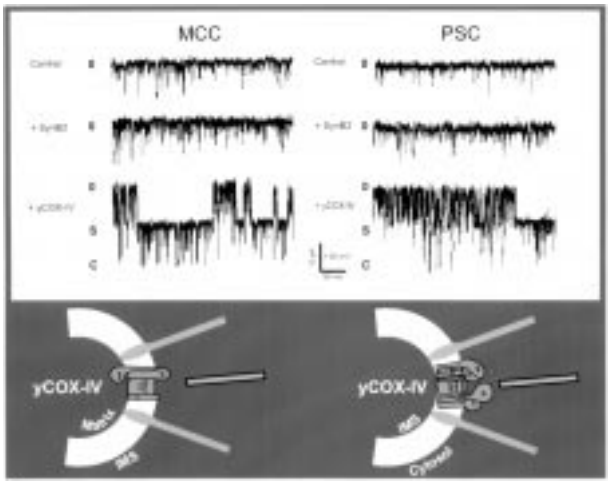


**Fig. 2.** The activities of two mitochondrial channels are modulated by synthetic targeting peptides. Typical current traces of MCC (left) and PSC (right) were recorded at 2 kHz from patches excised from proteoliposomes containing mitochondrial inner and outer membranes, respectively. Traces were obtained in the absence (control) and presence of targeting peptide (yCOX-IV from cytochrome oxidase subunit IV) or control peptide (SynB2) in symmetrical 0.15 M KCl, 5 mM HEPES, pH 7.4, under voltage-clamp conditions at 20 mV. O, S, and C indicate the open, substate and closed current levels. Illustration shows the orientations of the electrode tips and peptides to the Tim and Tom complexes.

## MCC AND PSC HAVE PORE DIAMETERS SUFFICIENT TO ALLOW POLYPEPTIDE TRANSLOCATION

MCC and PSC are high-conductance channels with similar single-channel behavior, as shown in the current traces of Fig. 2. While the sodium channel has a conductance of ~10 pS, the fully open states of MCC and PSC are ~1000 pS and their half-open states are ~500 pS in conductance (Lohret *et al.*, 1997; Jensen and Kinnally, 1997; Kinnally *et al.*, 1996; Lohret and Kinnally, 1995). These maximum conductances are consistent with pore diameters of ~2.7 nm for the open states of both channels, based on the method of Hille (1992). The single-channel behaviors of MCC and PSC, however, suggest the presence of double-barrel pores. In this case, the paired conductances of ~500 pS are consistent with a pore diameter of ~2 nm for each barrel. Importantly, this inferred pore diameter for MCC and PSC should be sufficient to allow the passage of unfolded polypeptides. Consistent with the predictions of a double-barrel pore structure based on single-channel behavior, single-particle image analysis of purified Tom complex shows two, and possibly three, apparent ~2 nm holes that may represent individual channel pores (Kunkele *et al.*, 1998). While the single-channel behaviors of MCC and PSC are similar, these two channel activities can be distinguished by antibodies (Lohret *et al.*, 1997), mutants (Lohret *et al.*, 1997), and protease sensitivity (unpublished data).

Several other mitochondrial channels, including the mitochondrial cento-picosiemen channel (mCS), the alkaline pH-activated anion and cation channels, and the ATP-sensitive $K^+$ channel, were eliminated as potential candidates for protein import pores since their estimated pore diameters would limit the passage of polypeptides. VDAC (voltage-dependent anion-selective channel), or mitochondrial porin, is located in the outer membrane and has a pore diameter of 2.5 to 3 nm (Guo *et al.*, 1995). However, there is no evidence that VDAC is associated with the Tim or Tom complexes and VDAC is not thought to play a role in protein import.

## CONDUCTANCES OF MCC AND PSC ARE MODULATED BY SYNTHETIC TARGETING PEPTIDES

The targeting regions of mitochondrial precursors are not conserved, but the domains are typically amphipathic α-helixes and have a net positive charge (Roise

and Schatz, 1988; Lemire *et al.*, 1989; Allison and Schatz, 1986). Synthetic targeting peptides modulate the conductance of ions through MCC and PSC in patch-clamp experiments, as shown in the current traces of Fig. 2. These peptides cause brief transient closures (flickering) of MCC and PSC that is reversible and voltage- and dose-dependent (Lohret *et al.*, 1997; Lohret and Kinnally, 1995). The number of rapid transitions from the normally occupied open state to the substate and closed conductance levels (seen as downward deflections in Fig. 2) dramatically increases in the presence of targeting peptides, *e.g.*, yCOX-IV$_{1-13}$ (first 13 amino acids of the precursor for cytochrome oxidase subunit IV). Targeting peptides, whose sequences support import of precursors into mitochondria, were found to induce flickering of MCC and PSC with the same efficacy as these targeting peptides competitively inhibit protein import. In contrast, peptides whose sequences do not support import, *e.g.*, SynB2, have no effect on MCC (Lohret *et al.*, 1997) or PSC (see Fig. 2). While PSC and MCC activities are specifically modulated by targeting peptides (Lohret *et al.*, 1997; Lohret and Kinnally, 1995; Kunkele *et al.*, 1998; Chich *et al.*, 1991; Fevre *et al.*, 1990, 1994; Henry *et al.*, 1989; Juin *et al.*, 1997), the activities of two other mitochondrial channel activities, mCS and VDAC, are not modified by these peptides (Lohret and Kinnally, 1995). Importantly, the modulation of MCC and PSC, by targeting peptides, has provided a means to evaluate the effects on these two channels of biochemical and genetic manipulations of the Tim and Tom complexes.

Current understanding of the routing of precursors to the different mitochondrial compartments predicts that presequences would interact with both membrane faces of the Tim complex and the cytosolic face of the Tom complex (Schatz and Dobberstein, 1996). While models of protein translocation do not require an interaction of presequences with the Tom complex on the intermembrane space side, it has been speculated that binding of presequences to an intermembrane space site on Tom40p might stabilize the precursor in the Tom complex. It is, therefore, interesting that applying targeting peptides to either side of the membranes, modify the single-channel behavior of both MCC and PSC (Lohret *et al.*, 1997). Hence, the flickering of MCC and PSC induced by targeting peptides in patch-clamp experiments may be a reflection of the interaction of the presequences with the Tim and Tom complexes, that is, the conformations necessary for the open states of MCC and PSC may destabilize upon binding of the targeting peptides. Alternatively, the flickering may reflect the transient occlusion of the pore of PSC and MCC during electrophoretic translocation of the peptides through their pores.

## FURTHER EVIDENCE THAT MCC IS THE PORE OF THE TIM COMPLEX

Several lines of evidence support the assignment of MCC as the protein import pore of the Tim complex of the inner membrane. Antibodies against Tim23p that inhibit protein import in mitoplasts also block the ion conductance through MCC (Lohret *et al.*, 1997). Preimmune serum, antibodies against VDAC, and antibodies against an iron sulfur protein of the inner membrane have no effects. Therefore, MCC is closely associated with Tim23p, a component of the import complex. A point mutation in Tim23 (*tim23.1*) that renders yeast mitochondria deficient in protein import also eliminates the modulation of MCC by targeting peptides (Lohret *et al.*, 1997, Ryan and Jensen, 1993; Emtage and Jensen, 1993). Since the mutation prevents incorporation of Tim23p into the Tim complex, the results suggest that Tim23p imparts peptide sensitivity on MCC, linking the channel to the import apparatus. If Tim23p constituted the pore of MCC, then depletion of Tim23p should have reduced the frequency of detecting MCC. However, no decrease in the detection of MCC was observed when the mitochondrial channel activity of the *tim23.1* strain was compared to the parental strain (Lohret *et al.*, 1997).

There are other observations that support the identification of MCC as the protein import channel of the inner membrane of mitochondria. As discussed above, the maximum pore diameter of MCC was estimated to be 2–3 nm (Kinnally *et al.*, 1996), which is large enough to accommodate an unfolded protein. Targeting peptides for mitochondria are positively charged and MCC is slightly cation selective (Lohret and Kinnally, 1995). Furthermore the pore of MCC is specifically opened and reversibly regulated by targeting peptides (Kushnareva *et al.*, 1999), as predicted by the general scheme for protein translocation.

MCC shares functional characteristics with other known or putative protein import channel activities. The predominant transition size (500 pS) of the MCC is the same as that of channels implicated in protein import in the endoplasmic reticulum and *E. coli* cell membrane (Simon and Blobel, 1991, 1992; Lohret *et al.*, 1997). The effect of targeting peptides on the *E.*

*coli* channel and MCC was voltage-dependent with similar dose dependencies (Simon and Blobel, 1992; Lohret and Kinnally, 1995). Finally, as noted above, the single-channel behavior of the MCC was identical to the PSC, the putative import channel of the outer membrane that has been studied in reconstituted systems (Lohret *et al.*, 1997). These results suggest that precursor proteins are translocated across the inner membrane through the pore of the MCC.

In efforts to determine the molecular identity of MCC, several knockout yeast strains and cell lines were examined for their MCC activity. These studies have eliminated several proteins as potential candidates for the pore of MCC, including the adenine nucleotide translocator (Lohret *et al.*, 1996), Tim23p (Lohret *et al.*, 1997), Tom20p, Tom70p, phosphate translocator, mitochondrial ABC transporter (unpublished results), VDAC (Lohret *et al.*, 1995), $F_0$ portion of ATP synthase, FeS protein, cytochrome *c* oxidase, cytochrome *b*, and the membrane-bound portion of NADH dehydrogenase (Murphy *et al.*, 1998).

## FURTHER EVIDENCE THAT PSC IS THE PORE OF THE TOM COMPLEX

The identification of PSC as the protein import pore of the Tom complex is supported by many of the experimental observations discussed above. Importantly, PSC activity is depleted by immunoprecipitation of solubilized membranes with antibodies against Tom40p (Juin *et al.*, 1997). Interestingly, channel activities are observed upon reconstitution of bacterially expressed Tom40p and of purified Tom complex in planar bilayers (Hill *et al.*, 1998, Kunkele *et al.*, 1998). While the single-channel conductances observed upon reconstitution of Tom40p and the Tom complex are significantly lower than that reported for of PSC, the kinetics, ion selectivity, and effects of targeting peptides are similar. The differences observed may have a physical basis, *e.g.*, intrinsic differences in membrane curvature for the planar bilayer and patch-clamp techniques. Alternatively, protein(s) in addition to Tom40p and those identified in the Tom complex may be required to attain the full conductance of PSC.

Like MCC, the estimated pore of PSC is large enough to accommodate an unfolded protein. The single-channel behavior of PSC in patch-clamp studies suggests the presence of double-barrel pores with $\sim$2 nm diameters (Fig. 2) and the single-particle analysis of the purified Tom complex shows a structure containing two to three pores with the same approximate diameters (Kunkele *et al.*, 1998). Finally, PSC activity is reversibly modulated by synthetic targeting peptides (Fevre *et al.*, 1994). In summary, these data strongly support the identification of PSC as the channel formed by Tom40p through which precursor proteins are translocated across the outer membrane.

## CONCLUSIONS

Our understanding of the mechanisms of protein translocation across membranes has advanced significantly in recent years. Many components of the mitochondrial import machinery have been discovered and their roles in protein transport have been unraveled by genetic and biochemical analysis. Electrophysiology, in particular patch clamping, has played a major role in the identification of MCC and PSC as the protein import channels of the Tim and Tom complexes. However, several important questions remain unanswered about protein translocation in mitochondria. In particular, what proteins make up the pore of the MCC in the Tim complex and what forces promote protein translocation through the Tom complex in the apparent absence of a motor?

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