Published in final edited form as:
Mol Cell Endocrinol. 2011 April 10; 336(1-2): 70–79. doi:10.1016/j.mce.2010.12.007.

Mitochondrial Protein Import and the Genesis of Steroidogenic Mitochondria

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Abstract

The principal site of regulation of steroid hormone biosynthesis is the transfer of cholesterol from the outer to inner mitochondrial membrane. Hormonal stimulation of steroidogenic cells promotes this mitochondrial lipid import through a multi-protein complex, termed the transduceosome, spanning the two membranes. The transduceosome complex is assembled from multiple proteins, such as the steroidogenic acute regulatory (STAR) protein and translocator protein (TSPO), and requires their targeting to the mitochondria for transduceosome function. The vast majority of mitochondrial proteins, including those participating in cholesterol import, are encoded in the nucleus. Their subsequent mitochondrial incorporation is performed through a series of protein import machineries located in the outer and inner mitochondrial membranes. Here we review our current knowledge of the mitochondrial cholesterol import machinery of the transduceosome. This is complemented with descriptions of mitochondrial protein import machineries and mechanisms by which these machineries assemble the transduceosome in steroidogenic mitochondria.

Keywords

Mitochondria; Protein import; Steroidogenesis; Cholesterol Transport; Translocator protein; Steroidogenic acute regulatory protein

1. Introduction

Steroid hormones produced by the adrenal glands and gonads of vertebrates are crucial determinants of a wide range of physiological functions, driving processes such as development, reproduction, and behavior. To generate steroid hormones, these tissues are equipped with a number of metabolic enzymes that metabolize precursor steroids into their

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products, with each tissue expressing an individual battery of steroidogenic enzymes (Payne and Hales, 2004). Despite the diversity of the steroid hormone family, all steroids derive from a single precursor, cholesterol, which is transformed to the steroid pregnenolone in the mitochondrial matrix by the cytochrome P450 cholesterol side chain cleavage enzyme, CYP11A1 (Jefcoate, 2002). By limiting access of the hydrophobic cholesterol molecule to CYP11A1, steroidogenic cells are able to control the amount of steroids they produce (Rone et al., 2009a). Much has been learned concerning the cellular expression and function of proteins involved in mitochondrial cholesterol movement and metabolism. However, in light of the vast body of knowledge accumulated over the past decade pertaining to mitochondrial protein import, comparatively little is known concerning their incorporation into the mitochondria. This review describes the mitochondrial steroidogenic and protein import machineries and subsequently focuses on how the interplay of the two generates steroidogenic mitochondria.

2. Steroid biosynthesis

Cholesterol serves as the metabolic precursor of all steroid hormones and is principally stored in the plasma membrane and lipid droplets of steroidogenic cells (Jefcoate, 2002; Mesmin and Maxfield, 2009). Steroid hormones are acutely synthesized in response to circulating peptide hormones, such as adrenocorticotropic hormone, luteinizing hormone, or chorionic gonadotropin. These hormones bind their cognate receptors on the surface of steroidogenic cells (adrenocorticotropic hormone for the adrenal and luteinizing hormone/chorionic gonadotropin for the gonad) and stimulate intracellular signaling cascades, of which the cAMP pathway, through protein kinase A (PKA), is the most prominent. Acutely, these events stimulate the flow of cholesterol to the inner mitochondrial membrane (IMM), where it is converted to pregnenolone by CYP11A1 (Jefcoate, 2002). In addition, hormonal and cAMP stimulation of steroidogenic cells is important for the chronic regulation of steroidogenesis, as continued exposure is necessary to ensure proper expression levels of steroidogenic proteins and steroidogenic metabolic flux (Simpson and Waterman, 1988).

Due to its hydrophobicity, cholesterol movement within the aqueous microenvironment of the cell requires the assistance of intracellular proteins (Mesmin and Maxfield, 2009). The translocation of cholesterol to the IMM is such a case, as the mitochondrion is a double-membrane organelle and cholesterol must traverse the aqueous intermembrane space (IMS) between the outer mitochondrial membrane (OMM) and the IMM where CYP11A1 resides. To accomplish this, steroidogenic cells possess a multicomponent protein machine recently named the transduceosome. This mitochondrial transduceosome is an ensemble of cytoplasmic and resident mitochondrial proteins, which receives hormonal signals and assists in the translocation of cholesterol across the IMS at contact sites between the IMM and OMM (Rone et al., 2009a) (Fig. 1). The core steroidogenic protein of the IMM, CYP11A1, associates with, and receives electrons from, the flavoprotein ferrodoxin reductase (also known as adrenodoxin reductase; (FdxR) and the iron/sulphur protein ferrodoxin (also known as adrenodoxin; Fdx) (Miller, 2005). Such steroidogenic cytochrome P450 enzymes and their associated electron transport chains have also been demonstrated to organize into higher order complexes (Praporski et al., 2009).

Also present in the IMM is the 35-kDa mitochondrial adenine nucleotide translocator (ANT). Though this protein has yet to have a definable role in mitochondrial cholesterol transport, immunoprecipitation experiments have demonstrated that ANT coprecipitates with two integral OMM proteins, presumably anchoring contact sites between the OMM and IMM. These OMM proteins were identified as the 31-kDa voltage-dependent anion channel (VDAC) and the 18-kDa translocator protein TSPO, previously named the peripheral-type benzodiazepine receptor (McEnery et al., 1992; Papadopoulos et al., 2006). These particular

OMM proteins have attracted considerable interest in steroidogenic research, as the benzodiazepine class of drugs is able to stimulate steroid biosynthesis (Krueger and Papadopoulos, 1990; Papadopoulos et al., 1991a) and the VDAC-TSPO pair is the site of benzodiazepine binding to the OMM (Garnier et al., 1994). TSPO itself is able to bind different classes of drugs with the capacity to stimulate steroidogenesis (Papadopoulos et al., 1991b; Rupprecht et al., 2009) in addition to cholesterol (Lacapere et al., 2001), and knockdown of TSPO ablates steroid biosynthesis in steroidogenic cells (Hauet et al., 2005; Papadopoulos et al., 1997).

In addition to the mitochondrial proteins making up core structural and enzymatic components of the transduceosome, cytoplasmic proteins are instrumental in regulating its assembly and cholesterol transport activity. Hormonal stimulation was found to promote the clustering of TSPO, and this clustering was correlated with steroidogenesis and could be suppressed by the PKA inhibitor H-89 (Boujrad et al., 1996). This suggests an active role for PKA in the assembly of the transduceosome. Yeast two-hybrid screening for additional cellular partners of TSPO yielded the acyl-coA binding domain family protein ACBD3/PAP7 (Li et al., 2001). ACBD3, which migrates from the Golgi to the mitochondria upon hormonal stimulation (Liu et al., 2006), is an A-kinase-associated protein and serves to scaffold the cytosolic PKA-RI subunits to the transduceosome (Li et al., 2001; Liu et al., 2006). Knockdown of ACBD3 suppresses hormone-induced steroidogenesis (Li et al., 2001) and the expression pattern of this protein is altered in the Carney complex neoplastic syndrome (Liu et al., 2003). An additional ACBD protein family member, ACBD1, participates in transduceosome function. Originally identified through its ability to displace benzodiazepine bound to GABA receptor sites in neurons (Costa and Guidotti, 1991), and hence originally named the diazepam binding inhibitor (DBI), ACBD1 acts on TSPO and stimulates steroidogenesis (Boujrad et al., 1993; Fan et al., 2010; Papadopoulos et al., 1991b)

Of the cytoplasmic transduceosome components, the protein that has garnered the greatest research attention is the steroidogenic acute regulatory protein, STAR. Mutations in STAR were found to underlie early childhood lethal congenital lipoid adrenal hyperplasia (lipoid CAH) in humans, a condition in which steroidogenesis is severely impaired and fatty deposits accumulate in steroidogenic cells (Lin et al., 1995; Miller, 2007). STAR was originally identified as a 30-kDa phosphoprotein located in the mitochondrial matrix and induced upon hormonal stimulation in adrenal and gonadal tissue (Pon et al., 1986; Pon and Orme-Johnson, 1986). Later, it was shown to be synthesized as a 37-kDa cytoplasmic protein with a mitochondrial presequence tag that is imported into the mitochondrial matrix and processed to a 30-kDa form (Clark et al., 1994; King et al., 1995). Cellular and biochemical work indicate that STAR functionally promotes steroidogenesis at the OMM and is inactive in the matrix (Bose et al., 2002; Miller, 2007), making the role of STAR import and processing unclear. Questions remain concerning the molecular factors regulating STAR's mitochondrial import, a topic that will be discussed below.

Though gene expression of the transduceosome components has been the focus of numerous studies [see (Lavoie and King, 2009) and (Batarseh and Papadopoulos, 2010) for recent reviews], much less is known about the way cells target the transduceosome components to their proper location in the mitochondria, a factor critical to their function in steroidogenesis, as demonstrated for CYP11A1 (Black et al., 1994). To ensure a proper background for our discussion of mitochondrial targeting of the different transduceosome components, the following section comprises a brief review of our current understanding of mitochondrial protein import, limiting our discussion to those aspects of mitochondrial protein import implicated in the assembly of the transduceosome. For more detailed information

concerning mitochondrial protein import, readers are referred to some excellent recent reviews (Chacinska et al., 2009; van der Laan et al., 2010).

3. Mitochondrial Protein Import

3.1. Mitochondrial protein import and chaperones

Mitochondria are proposed to have arisen from the symbiotic partnering of a proto-eukaryotic and a proto-α-bacterium (Cavalier-Smith, 2009; Gray et al., 1999). Though the details of this symbiotic relationship are a topic of much contention, the proposal itself has garnered support, in large part due to the presence of an independent mitochondrial genome separate from the one present in the eukaryotic nucleus (Baker et al., 1996). Despite the presence of the mitochondrial genome, the vast majority of mitochondrial proteins are encoded in the nucleus and transcribed in the cytosol as mitochondrial preproteins, which contain sequence information that targets them to the mitochondria (Chacinska et al., 2009). The majority of mitochondrial precursor proteins are targeted to the mitochondria through an N-terminal stretch of 15–55 amino acids, termed the presequence, which is cleaved upon import (Vogtle et al., 2009). Mitochondrial preproteins are targeted to one of four mitochondrial subcompartments, namely the matrix, the IMM, the IMS, or the OMM (Chacinska et al., 2009). As isolated mitochondria are competent to import *in vitro* transcribed and translated proteins (Maccecchini et al., 1979), the current consensus is that mitochondrial protein import primarily occurs post-translationally. This is in sharp contrast to the paradigmatic targeting of proteins to the endoplasmic reticulum, which occurs co-translationally (Schwartz, 2007). As such, there is a need for cytosolic chaperones to maintain mitochondrial preproteins in their unfolded, import-competent state, and to assist with the early stages of import into the mitochondria.

Mitochondrial protein import initiates in the cytosol with the recognition of the preprotein by cellular chaperones. The most prominent of these chaperones are Hsc70 (heat shock cognate protein of 70-kDa chaperone) and HSP90 (heat shock protein 90), which function to stabilize the preprotein, protecting it from aggregation and degradation (Fan et al., 2006; Voos and Rottgers, 2002; Young et al., 2003). Hsc70 binds to and shields preprotein internal hydrophobic sequences, whereas HSP90 has a high affinity for the features of non-native or destabilized proteins (Scheufler et al., 2000). Hsc70 and HSP90 subsequently target the preprotein to the mitochondria, docking with OMM import receptors (Young et al., 2003). Upon binding, the chaperones are released by ATP hydrolysis and the preprotein is transferred to the mitochondrial import machinery (Young and Hartl, 2000; Young et al., 2003).

Import of proteins into the mitochondria is an intricate process, relying on the coordination of import machinery localized at each membrane, namely the Translocase of the Outer Mitochondrial Membrane (TOM) complex for the OMM, and the Translocase of the Inner Mitochondrial Membrane (TIM) complex for the IMM. Of late, the field expanded greatly with the identification of the sorting and assembly machinery (SAM) in only the last 10 years (Wiedemann *et al.*, 2003).

3.2. Translocase of the Outer Membrane (TOM) Complex

The receptors for the chaperone-carried preproteins comprise components of the TOM complex in the OMM. The TOM Complex is a 440-kDa protein complex which functions as a general import pore (van Wilpe et al., 1999; Wiedemann et al., 2004). TOM is assembled from six core proteins: Tom40, the channel for preprotein import; Tom20 and Tom22, specialized receptors for import; Tom6, which stabilizes the TOM complex; Tom7, which favors TOM dissociation; and Tom5, which assists with the transfer of preproteins (Dietmeier et al., 1997; Kato and Mihara, 2008; Model et al., 2001; Schmitt et al., 2005;

Yano et al., 2004). Tom40, a β-barrel protein, serves as the principal channel for the import of polypeptides while Tom20 binds mitochondrial presequences and shuttles the proteins to Tom40, with the assistance of Tom22 and Tom5 (Abe et al., 2000). Alternatively, preproteins without presequences and their chaperones associate with the accessory Tom70 receptor, which facilitates transfer to the TOM complex (van Wilpe et al., 1999). Upon translocation of the protein through the Tom40 pore, the presequence binds to Tom7, and then binds to the IMS domain of Tom22. At this point, the protein is present in both the cytosol and the IMS of the mitochondria and targeting information nascent in the polypeptide targets it to its next site of translocation (Chacinska *et al.*, 2009).

3.3. Sorting and Assembly Machinery (SAM) Complex

OMM β-barrel proteins, such as Tom40, are imported into the OMM through the action of the TOM complex and subsequently inserted into the membrane through a distinct OMM import assembly, the SAM complex. In yeast, the SAM complex consists of three proteins: Sam35, the initial receptor for the preprotein; Sam50, a β-barrel import pore; and Sam37, which functions to integrate the protein into the OMM (Pfanner *et al.*, 2004). Upon the presence of the preproteins in the IMS, the chaperone complex Tim8 – Tim13 [or the distinct Tim9 – Tim12 pair (Chan and Lithgow, 2008)] binds to the preprotein and transfers it to the SAM receptor protein Sam35 (Chan and Lithgow, 2008). Sam35 transfers the preprotein to Sam50; with its release into the lipid membrane regulated by Sam37 (Chan and Lithgow, 2008; Kutik et al., 2008), though the exact mechanism of SAM-mediated protein insertion into the OMM is still unknown (Becker et al., 2008; Kutik et al., 2008)

3.4. α-Helical Protein Incorporation into the OMM

Though the most prevalent protein in the OMM is the β-barrel protein VDAC, the majority of proteins present in the OMM are α-helical. Simple OMM proteins that span the membrane in one α-helix, such as Tom20 or Tom22, have been shown to have mitochondrial targeting sequences at either the N- or C-terminus (Rapaport, 2003). For these proteins, several paths of import have been identified, with the transmembrane region and flanking positive amino acids functioning as a putative targeting sequence (Ahting et al., 2005; Rapaport, 2005). N-terminally-targeted proteins utilize the Tom40 pore but the TOM complex receptors Tom20 and Tom70 have been shown not to be utilized (Ahting et al., 2005; Van den Berg et al., 2004) (Fig. 2). Mim1 has been shown to promote the insertion of proteins containing N-terminus targeting sequences, such as Tom20 (Dimmer and Rapaport 2010). A mechanism for C-terminally-anchored OMM proteins has not been identified, though both lipid composition and the SAM complex have been implicated (Kemper et al., 2008; Setoguchi et al., 2006; Stojanovski et al., 2007). Proteins that span the OMM with multiple transmembrane domains (MTD) have targeting sequences found throughout the protein (Otera *et al.*, 2007). The core TOM complex is not necessary for import of MTD proteins, but the TOM receptor Tom70 appears to be critical for import (Rone *et al.*, 2009b).

3.5. Translocase of the Inner Membrane (TIM) Complex

Following TOM translocation to the IMS, there are two primary pathways for preprotein import into the IMM that involve Tim22 and Tim23, respectively (Fig. 3). Tim22 plays a role in the import of the IMM metabolite carrier proteins, which do not contain an explicit targeting sequence, while Tim23 functions in the import of proteins containing a presequence targeted for the matrix (van der Laan et al., 2010). Tim23 is also instrumental in the import of IMM proteins that span the membrane with a single α-helix (van der Laan et al., 2010). The pathway for import through Tim22 begins when Hsc70 and HSP90 target the protein to the OMM where they pass through the TOM complex pore (Brix et al., 1999;Stan et al., 2003). Upon import into the IMS, the Tim9 – Tim12 pair binds to the protein and shuttles it to the Tim54 receptor (Endres et al., 1999;Vasiljev et al., 2004). Tim54 transfers

the preprotein to the Tim22 pore, which then releases the protein into the IMM (Chacinska et al., 2009).

The Tim23 protein complex is composed of three proteins regulating the import of preproteins into the IMM or the matrix: Tim50, Tim23, and Tim21 (Mokranjac and Neupert, 2010). Upon import through the TOM pore, Tim50 binds and transfers the presequence-containing preprotein from Tom22 in the IMS to Tim23, the IMM protein import pore (Tamura et al., 2009). Tim21 competes with the presequence binding domain of Tom22, facilitating the transfer of the preprotein to Tim23 (Chacinska et al., 2005). In the case of IMM proteins, a hydrophobic stretch in the preprotein (sorting signal) stalls import upon incorporation into the Tim23 pore, signaling insertion of the protein into the IMM through lateral diffusion from the TIM pore (Popov-Celeketic et al., 2008). The non-hydrophobic portion of the preprotein is imported into the matrix through the preprotein assembly and machinery (PAM) complex, where the targeting sequence is usually cleaved. The interaction between Tim23 and the PAM complex also supports the translocation of preproteins targeted to the matrix.

3.6. Preprotein Assembly and Machinery (PAM) Complex

The positive charge of the mitochondrial preprotein must overcome a steep thermodynamic barrier generated by the proton gradient in the IMS to reach the mitochondrial matrix (Shariff et al., 2004). Such an energetic contribution is supplied by the PAM complex, which traps and pulls the preprotein into the mitochondrial matrix in an ATP-dependent manner (Krayl et al., 2007). The PAM complex is composed of Tim44, mtHSP70, Pam16, and Pam18 (Fig. 3) (van der Laan et al., 2010). Tim44 is anchored to the matrix side of the IMM via its N-terminus. The membrane-facing side of Tim44 interacts with the Tim23 pore while the matrix-facing side of Tim44 interacts with mtHSP70, which physically ratchets the protein into the matrix (D'Silva et al., 2004; Rassow et al., 1994; Tomkiewicz et al., 2007). Pam16 and Pam18 subsequently interact with mtHSP70 to assist in the regulation of ATP hydrolysis, modulating the rate of protein import (Li et al., 2004).

3.7. Mitochondrial protein processing

In addition to importing protein across their double membranes, mitochondria process and remodel many of these proteins before utilization. It is also important for mitochondria to degrade misfolded or unwanted proteins. To this end, mitochondria contain multiple peptidases and proteases, primarily localized to the IMM and the matrix, which target proteins resident in the IMS and matrix. Upon import into the matrix, the presequence of many proteins targeted to the matrix is removed by the mitochondrial processing peptidase (MPP) (Gakh et al., 2002). Retention of the presequence appears to destabilize the protein conformation, making it susceptible to increased proteolysis by mitochondrial peptidases (Mukhopadhyay et al., 2007). MPP is a heterodimer composed of α-MPP and β-MPP (Kalousek et al., 1993) located in the mitochondrial matrix. It is a metallopeptidase inhibited by low pH and metal chelators (Miura et al., 1982). MPP recognizes the positively charged α-helix of presequences in an extended conformation (Taylor et al., 2001) and cleaves the presequence two residues after an arginine residue present in three sequence motifs (Hendrick et al., 1989; Schneider et al., 1998). For some mitochondrial preproteins, the presequence is sequentially processed by MPP and an additional matrix metallopeptidase, Oct1 (Branda et al., 1999), which cleaves the remaining presequence eight amino acids following the MPP cleavage site. A third peptidase complex, the inner membrane peptidase (IMP), assists in the maturation of IMS proteins. IMP proteins are bound to the IMM and project into the IMS (Jan et al., 2000) and require divalent cations and acidic phospholipids for activity (Schneider et al., 1991).

A variety of mitochondrial members of the AAA+ (ATPases associated with various cellular activities) superfamily have been identified (Gakh et al., 2002; Truscott et al., 2010). Though these enzymes appear to function principally in ATP-dependent protein quality control, several have been implicated in the import and processing of mitochondrial preproteins (Rainey et al., 2006). The Lon and CplX proteases are located in the mitochondrial matrix (Kang et al., 2002; Wang et al., 1993), and the Lon protease is considered to play a major role in mitochondrial protein clearance (Major et al., 2006; Ondrovicova et al., 2005). The IMM-bound proteases m-AAA (matrix-AAA) and i-AAA (intermembrane space-AAA) target proteins present in the matrix and IMS, respectively (Arlt et al., 1996; Leonhard et al., 1996). These proteases are considered the principal players in quality control of unassembled and misfolded proteins in the IMM (Koppen and Langer, 2007), though m-AAA has been noted be involved in the maturation of several mitochondrial proteins (Nolden et al., 2005; Rainey et al., 2006).

4. Mitochondrial Import of Cholesterol Transduceosome Proteins

As described above, the import of mitochondrial proteins occurs through a series of elaborate and tightly regulated processes. In the subsequent sections, we describe the interplay of these protein import pathways and our knowledge of the import of various protein components of the transduceosome, namely VDAC and TSPO on the OMM, and CYP11A1, Adx, and AdxR in the mitochondrial matrix.

4.1. Voltage-Dependent Anion Channel (VDAC)

VDAC is an OMM β-barrel protein involved in the regulation of ions and small molecules across the OMM, influencing multiple cellular processes, including apoptosis and energy transduction (Vyssokikh and Brdiczka, 2003). VDAC has been shown to play an important role in steroidogenesis, interacting with TSPO at the OMM in both helping to anchor the transduceosome to the OMM and assisting with the import of STAR (Bose et al., 2008; Liu et al., 2006).

The structure, but not homology, of VDAC is similar to Tom40, in that they both are β-barrel proteins that span the OMM 19 times (Zeth, 2010). Studies in the fungi *Neurospora crassa* and *Saccharomyces cerevisiae* demonstrated that an internal targeting sequence of VDAC interacts with both Tom20 and Tom22 and facilitates import by the TOM complex (Krimmer et al., 2001). Additional import partners were identified by OMM permeabilization, promoting the loss of IMS space proteins and revealing a critical role for the Tim8 – Tim13 heterodimer in VDAC import (Krimmer et al., 2001). Interaction of VDAC with these IMS proteins promotes transfer to Sam35 and its subsequent incorporation into the OMM by Sam50 (Kozjak et al., 2003; Milenkovic et al., 2004). Removal of the C-terminus of VDAC inhibits its maturation, suggesting the presence of a signal in this region for the targeting or binding of VDAC to the SAM complex (Kutik et al., 2008).

Knockdown of Tom40 in HeLa cells by shRNA decreased VDAC import, further accentuating the importance of the TOM complex for VDAC import in mammalian cells (Kozjak-Pavlovic et al., 2007). Reduction of the SAM complex component Sam50 in HeLa cells also significantly reduced the import of VDAC, though this reduction correlated with reductions in the SAM complex proteins Metaxin1 (mammalian Sam37) and Metaxin2 (mammalian Sam35) (Armstrong et al., 1997; Armstrong et al., 1999; Kozjak-Pavlovic et al., 2007). Knockdown of Metaxin2 revealed the importance of smaller SAM components for VDAC import, as VDAC levels were decreased by 50% in the OMM of Metaxin2 knockdown cells with only 10% assembling into a mature complex (Kozjak-Pavlovic et al., 2007). Though the targeting sequence for mammalian VDAC remains unknown, the TOM

and SAM complexes have a demonstrably critical, and phylogenetically conserved, role for VDAC import and assembly in the OMM.

4.2. Translocator Protein (TSPO)

TSPO is an 18-kDa OMM protein with a predicted structure of five transmembrane α-helices, (Culty et al., 1999; Joseph-Liauzun et al., 1998) a prediction recently supported by cryoelectron microscopy (Korkhov et al., 2010). Like all OMM proteins, TSPO does not possess an explicit mitochondrial targeting presequence. Generation of a series of TSPO deletion mutants revealed that the C-terminal half of the protein is necessary and sufficient to target TSPO to the OMM (Rone et al., 2009b). Analysis of these peptides revealed the presence of a Schellman motif between amino acid 103–109, which functions to terminate one α-helix, form a short hairpin loop, and begin another α-helix (Viguera and Serrano, 1995). Complete removal of this motif or mutation of a critical central glycine also resulted in inhibition of TSPO targeting and insertion into the OMM (Rone et al., 2009b). From these studies, it was proposed that TSPO functions as a C-terminally-anchored protein that requires a Schellman motif for correct protein folding and chaperone binding.

TSPO's mitochondrial targeting and import are assisted by cytosolic chaperones, specifically Hsc70 and HSP90 (Rone et al., 2009b). Upon arrival of TSPO at the OMM, the chaperones interact with Tom70 in an ATP-dependant manner, releasing an import-competent TSPO (Otera et al., 2007; Rone et al., 2009b). Interestingly, upon its release, TSPO import does not require the core TOM complex proteins Tom20, Tom22, or Tom40, as determined by RNAi knockdown (Otera et al., 2007). Though the core TOM complex is not necessary for TSPO import, these findings do not discount the possible contributions of IMS import proteins. Removal of the IMS protein Tim8 by hypotonically stressing the mitochondria decreased TSPO import without affecting OMM (Tom22) or IMM (Su9-DHFR) protein import (Otera et al., 2007). Additional machinery contributing to TSPO import was determined using RNA silencing. Though knockdown of the SAM complex component Sam50 did not affect TSPO levels (Otera et al., 2007), the Sam37 homolog, Metaxin1, was found to be necessary (Rone et al., 2009b). Sam37 has been demonstrated to mediate the insertion of proteins carrying C-terminal targeting information into the OMM (Stojanovski et al., 2007), and in light of the importance of TSPO's C-terminus for targeting, these findings suggest a principal role of Metaxin1 for TSPO membrane insertion.

4.3. Cholesterol Side-Chain Cleavage Cytochrome P450 (CYP11A1)

CYP11A1 comprises the core metabolic enzyme of the transduceosome, catalyzing the conversion of cholesterol to pregnenolone through cleavage of the aliphatic side chain of cholesterol (Payne and Hales, 2004). Human CYP11A1 is encoded as a 521-amino acid polypeptide with a 39-amino acid mitochondrial presequence, which is cleaved upon import (DuBois et al., 1981). Point mutation of the three positive amino acids in the presequence greatly reduced the efficiency of import into the matrix, confirming the necessity of positive amino acids for preprotein import (Nabi et al., 1983). However, the association of CYP11A1 with the matrix was not affected by the presence of the presequence, as inhibition of presequence processing by the metal chelator *o*-phenanthroline did not affect CYP11A1 mitochondrial integration and enzymatic activity (Ou et al., 1986). Studies of CYP11A1 membrane topology using Na_2CO_3 treatment of isolated mitochondria revealed that CYP11A1 tightly associates with mitochondrial membranes and has its N-terminus protected from trypsin proteolysis, suggesting that the N-terminus anchored the protein to the IMM (Ou et al., 1986). Despite these proteolytic protection studies, sequence analyses could not identify the hydrophobic sequence of the mature protein for IMM insertion (Nelson and Strobel, 1988), suggesting that CYP11A1 is fully imported into the mitochondrial matrix and associated peripherally with the IMM. Modeling studies of

CYP11A1 have proposed that membrane association occurs through an F-G loop and residues surrounding the α-helix of the protein (Headlam et al., 2003; Storbeck et al., 2007), a hypothesis supported by mutational analyses (Pikuleva, 2004; Pikuleva et al., 2008).

Multiple studies examining the molecular mechanisms of CYP11A1 import have utilized yeast as a model system. Upon import of the mammalian CYP11A1 into yeast mitochondria, the protein is quickly degraded, with only a small sample functionally integrating into the IMM (Minenko et al., 2008; Savel'ev et al., 1998). To determine the mechanism of CYP11A1 import and further confirm where it is localized in the mitochondria, the 39-amino acid targeting sequence of CYP11A1 was removed and hybrid proteins were created, each with one of six differential mitochondrial targeting sequences, which would be imported into the mitochondria by one of the import machineries. Functional import was measured by measuring the activity of CYP11A1 (Minenko et al., 2008). It was determined that the AAC-CYP11A1 complex, a fusion of CYP11A1 with the full size adenine nucleotide translocase component (AAC), was the most functional hybrid when targeted and imported into the mitochondria via the Tim22 complex. Decreased enzymatic activity was seen when the complex was imported completely into the matrix and then reassembled through the PAM complex, as seen with the preadrenodoxin (Padx)-CYP11A1 complex (Minenko et al., 2008). Rather than indicating the requirement of the Tim22 pathway for import, these results suggest that a tight association with the IMM needs to be maintained for the functionality of CYP11A1. As cholesterol is a hydrophobic molecule, this tight association would provide a mechanism for the transfer of cholesterol to CYP11A1. The probable mechanisms of import of CYP11A1 into the matrix would involve the Tim23-PAM pathway. However, how this tight association is maintained during import or re-established after import is not understood.

4.4. Ferredoxin (Fdx) and Ferredoxin Reductase (FdxR)

CYP11A1 activity is regulated by the rate of electron transfer from the matrix proteins Fdx and FdxR (Miller, 2005). Fdx served as one of the first models of mitochondrial protein import, with initial work on Fdx import beginning in 1980. Padx, the precursor for Fdx, is synthesized in the cytosol at approximately 20-kDa and, upon import, its presequence of 58 amino acid is cleaved, resulting in a 14-kDa protein (Nabi and Omura, 1980). Studies in *E. Coli* demonstrated that the presence of the presequence did not alter the correct folding pattern of the protein or its ability to bind to FdxR, but did decrease its ability to bind to CYP11A1 (Goder et al., 1998). Targeting of Padx to the mitochondria occurs through either HSP70 or the mitochondrial import stimulation factor (MSF) (Hachiya et al., 1994; Hachiya et al., 1995), though in early experiments it was shown that ribosomes synthesizing Padx were also able to bind to the OMM (Nabi and Omura, 1983). This suggests that both cytosolic synthesis and mitochondrial localized synthesis could lead to import and cleavage of Adx into the mitochondria (MacKenzie and Payne, 2004). FdR is also imported via a mitochondrial targeting sequence, of which 32 amino acids are cleaved from the 462-amino acid protein after import (Sagara et al., 1987), though no further examination of its import has been completed. As these proteins are localized in the mitochondrial matrix where they interact with CYP11A1, it is possible that they are imported through the Tim23 import complex to the IMM membrane where the PAM complex would assist with import and proper folding in the matrix.

4.5. Steroidogenic Acute Regulatory Protein (STAR)

STAR received a great deal of research attention after the discovery that its mutation results in lethal congenital adrenal lipoid hyperplasia (CAH), a disease characterized by the inability to synthesize steroids (Lin et al., 1995; Miller, 2005). Deletion of *Star* in mice resulted in an adrenal phenotype similar to that observed in the human disease, although

gonadal function was affected to a lesser extend (Caron et al., 1997) STAR is expressed as a 37-kDa preprotein composed of a cholesterol-binding START (STAR-related lipid transfer) domain preceded by a mitochondrial presequence tag (Clark et al., 1994; Strauss, III et al., 2003; Tsujishita and Hurley, 2000). In rodents, the STAR preprotein is targeted to mitochondria and proteolytically processed to yield a 32- and a final 30-kDa protein, which is located in the mitochondrial matrix (Epstein and Orme-Johnson, 1991; Stocco and Sodeman, 1991). Mutational analysis suggests the presence of proteolytic cleavage sites at positions 39/40 and 55/56 of the bovine STAR protein, with similar predicted sites existing in other mammalian STAR homologs (Yamazaki et al., 2006). Upon reaching the matrix, the 30-kDa STAR is degraded, with a half-life of four to five hours (Granot et al., 2003). Inside the matrix, STAR protein levels are controlled by the ATP-dependent Lon protease, though this regulation is undoubtedly complex as Lon proteases degrade STAR within five minutes in an *E. coli* model system (Granot et al., 2007). It is also unclear which mitochondrial proteases process STAR. Processing of STAR from a 37-kDa to 32-kDa to a 30-kDa protein was suggested by studies using mitochondrial toxins to impede protein import and proteolysis. The protonophore CCCP, which disrupts the mitochondrial membrane potential, stabilized 37-kDa STAR, while 1,10-orthophenanthroline, a transition metal chelator proposed to inactivate metal-dependent proteases, stabilized the 32-kDa form (Artemenko et al., 2001). As membrane potential disruption blocks preproteins from inserting into, but not reaching, the IMM (van der Laan et al., 2010), these authors proposed that an IMS protease processes STAR from 37- to 32-kDa, which is further processed by a MPP upon crossing the IMM (Artemenko et al., 2001). The IMP and i-AAA discussed above are both metallopeptidases facing the IMS, making them excellent candidates for STAR's processing protease, though further work will be required to confirm this hypothesis.

Though STAR contains a classical mitochondrial presequence and is thus presumed to be imported through the TOM-TIM complexes of the OMM and IMM, precise molecular details are lacking. This gap in our knowledge is especially pertinent in light of recent work with the transduceosome components TSPO and VDAC. TSPO knockdown studies examining the functional relationship between STAR and TSPO in the stimulation of steroidogenesis demonstrated that STAR's steroidogenic ability is dependent on the presence of TSPO in MA-10 mouse Leydig tumor cells (Hauet et al., 2005). Intriguingly, decreasing TSPO expression reduced levels of the 30-kDa protein, but increased levels of 37-kDa STAR. Similar results were obtained with a peptide antagonist of TSPO function (Gazouli et al., 2002; Hauet et al., 2005), suggesting a role for TSPO in STAR's import and processing. This work has been more recently complemented by studies demonstrating the necessity of VDAC in STAR's steroidogenic function (Bose et al., 2008). In these investigations, VDAC knockdown reduced STAR expression, and acute blockage of VDAC with polyanion inhibitors prevented the conversion of the 32-kDa form to the 30-kDa matrix form. Collectively, these studies demonstrate an integral correlation between STAR's steroidogenic function and its mitochondrial import. As shown schematically in Figure 4, this conclusion raises interesting questions for study of the interrelationship between mitochondrial protein and cholesterol import, questions currently under investigation in our laboratory.

5. Summary and Conclusions

The large majority of mitochondrial proteins are encoded in the nucleus, translated in the cytoplasm, and imported into the mitochondria. The double mitochondrial membranes create four mitochondrial subcompartments, in addition to the cytoplasm, which differ according to their hydrophilic or hydrophobic nature: a hydrophobic outer mitochondrial membrane, a hydrophilic intermembrane space, a hydrophobic inner mitochondrial membrane, and a hydrophilic mitochondrial matrix. Proteins destined for these mitochondrial compartments

also possess chemical natures similar to their destination (i.e., hydrophobic residues are targeted to hydrophobic membrane compartments). As a consequence, proteins require cellular machinery in the OMM and IMM to allow their transport to their particular mitochondrial localization through compartments that they could not freely cross. A similar situation is observed with the lipid cholesterol, where a multi-protein machine termed the transduceosome translocates cholesterol to the IMM and metabolizes it to the steroid pregnenolone. Core members of the transduceosome, such as TSPO, VDAC, and CYP11A1, are encoded in the nucleus and must be transported into the mitochondria for activity. Hormone stimulation induces the translocation of the ACBD3/PAP7 protein from the Golgi apparatus to the mitochondria, where it serves to scaffold the cytosolic PKA-RI subunits. Moreover, a critical regulatory element, STAR, is a mitochondria-targeted protein whose activity is increased upon PKA-mediated phosporylation, underscoring the tight interplay between mitochondrial protein and lipid import in steroidogenic mitochondria. This interplay is a burgeoning field of research for protein import, as it has recently been appreciated that mitochondrial phospholipid synthesis is dependent upon the protein import machinery (Osman et al., 2009) and lipids critically regulate the function of translocase complexes (van der Laan et al., 2007). Finally, the observation that the activity of the transduceosome components TSPO and VDAC regulate the import and processing of STAR (Bose et al., 2008; Hauet et al., 2005) further suggests a connection between mitochondrial lipids and protein import. The mechanistic understanding of this process will increase our understanding of mitochondrial biogenesis.

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01 ES07747), the Canadian Institutes of Health Research (MOP102647), a Canada Research Chair in Biochemical Pharmacology to V.P., and a postdoctoral fellowship from CIHR (TGF36110) to A.M.

Abbreviations

Fdx	ferredoxin
FdxR	ferredoxin reductase
IMM	inner mitochondrial membrane
IMS	intermembrane space
MPP	mitochondrial processing peptidase
OMM	outer mitochondrial membrane
PAM	preprotein assembly and machinery
PKA	protein kinase A
SAM	sorting and assembly machinery
STAR	steroidogenic acute regulatory protein
TIM	translocase of inner mitochondria
TOM	translocase of outer mitochondria
TSPO	translocator protein
VDAC	voltage-dependent anion channel

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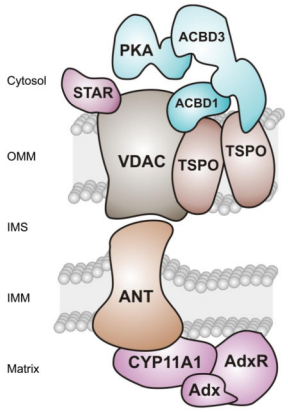

Figure 1. Transduceosome formation at the mitochondria upon hormonal stimulation

Cytosolic transduceosome proteins PKA, ACBD3, and ACBD1 are shown in blue while mitochondrial matrix proteins STAR, CYP11A1, Adx, and AdxR are shown in purple. OMM transduceosome proteins VDAC and TSPO, and the IMM protein ANT, are represented in brown.

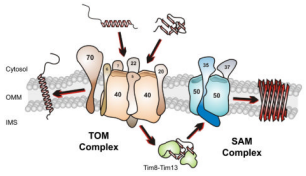

Figure 2. Mechanisms of protein import into the OMM

Import of α-helical proteins into the OMM requires the TOM complex, shown in orange, though exact mechanisms vary by targeting mechanisms located on the protein. Import of β-barrel proteins into the OMM requires both the TOM complex and the SAM complex, shown in blue. Transport of the preprotein from the TOM complex to the SAM complex uses the Tim8-Tim13 chaperone complex, shown in green.

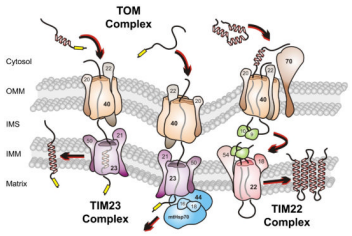

Figure 3. Mechanisms of protein import into the IMM

Alpha-helical IMM proteins are imported via Tim23, shown in purple. Import of matrix proteins requires Tim23 and the additional PAM complex and mtHSP70 (*blue*). Import of multi-membrane spanning IMM proteins is accomplished through the small Tim proteins Tim9-Tim10, shown in green, which transport the preprotein to Tim22, shown in pink. TOM complex is shown in brown, Tim23 complex is shown in purple, and Tim22 complex is shown in pink.

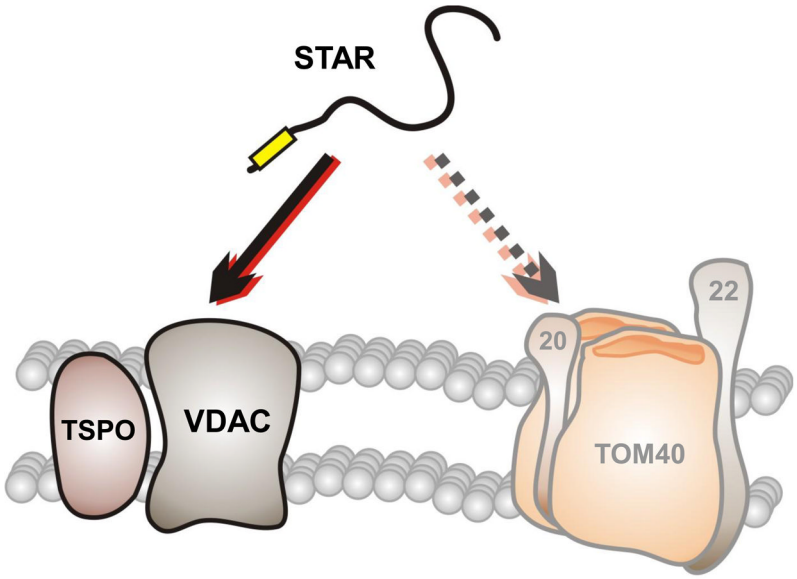

Figure 4. STAR protein import and its relationship to the transduceosome in a working model of OMM proteins

Experimental evidence demonstrates that TSPO and VDAC are critical for the mitochondrial import and processing of the cytoplasmic STAR protein. Though the mitochondrial TOM (faded orange) and Tim complexes (not shown) are implicated in STAR import by its mitochondrial localization and presequence tag, direct experimental evidence of association with the mitochondrial protein import machinery is lacking. See text for further details.