ROLE OF PROTEIN-PROTEIN INTERACTIONS IN MITOCHONDRIAL PROTEIN IMPORT, CHOLESTEROL TRANSPORT AND STEROID BIOSYNTHESIS

A Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry and Molecular & Cellular Biology

By

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Thesis Advisor: Vassilios Papadopoulos, DPharm, PhD.

ABSTRACT

Steroid synthesis is initiated by the transfer of cholesterol to the inner membrane of the mitochondria where the conversion of cholesterol to pregnenolone occurs through the C27 cholesterol side chain cleavage cytochrome P450 enzyme (P450scc; CYP11A1). The rate of steroidogenesis is not regulated by the activity of CYP11A1 but by the availability of substrate. As the process of trafficking cholesterol to the mitochondria can occur through a series of cytosolic and mitochondrial proteins and vesicular interactions, we propose that this delivery of cholesterol into the mitochondria occurs through specific protein-protein interactions that drive steroidogenesis.

This hypothesis was explored using MA-10 mouse Leydig cells, which undergo steroidogenesis to produce progesterone. It was identified through cross-linking studies that a protein complex formed at the outer mitochondrial membrane (OMM) upon hormonal stimulation. This complex consisted of mitochondrial protein translocator protein (TSPO, 18 kDa), Golgi protein PBR Associated Protein 7,
(PAP7), cytosolic protein PKA-RIα, and mitochondrially targeted Steroidogenic Acute Regulatory protein (StAR). TSPO assists with the translocation of cholesterol from the OMM to the IMM and is an integral OMM protein; therefore, we decided to study the mechanisms of its import and integration into the OMM. We identified that the C-terminus and amino acids #103-109 functioned in targeting TSPO to the mitochondria through the assistance of heat shock proteins (HSP). Translocase of Outer Mitochondrial (TOM) Membrane protein Tom70 interacted with the HSP’s in an ATP-dependant manner to import TSPO with the aid of the Metaxin1. From these findings, we went on to study the protein-protein interactions formed by TSPO in the mitochondria. Upon hCG treatment TSPO undergoes polymerization, identification of a shift in TSPO molecular weight and proteins that associate with TSPO was observed through Blue Native-PAGE and 2D-SDS PAGE immunoblot (western) analysis after hCG treatment in MA-10 cells. From this we identified a BN-PAGE shift in CYP11A1 from a lower to a higher molecular weight complex, suggesting it interacts specifically with the polymerized TSPO, forming of a steroidogenic protein complex.

In summary, these studies demonstrate that delivery of cholesterol into the mitochondria and thus steroidogenesis are driven by a series of protein-protein interactions.
ACKNOWLEDGEMENTS

As another wise Missourian once said, “Keep away from people who try to belittle your ambitions. Small people always do that, but the really great make you feel that you, too, can become great.” I would like to think my thesis mentor Dr. Vassilios Papadopoulos for always making me feel that I can become, if I was not already, great. If it was not for his guidance, support and understanding this thesis would not have been possible.

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Thanks and gratitude goes out to my thesis committee members, Dr. Elliot Crooke, Dr. Paul Roepe, Dr. Richard Youle, and Dr. Jason Young for all of their advice and guidance during these past seven years.

Words alone are not possibly enough to thank all my family and friends. To my parents, Lewis and Martha, thank you for your continued love and support. Without the values and morals you instilled in me I could not have become the person I am today. To Justin, Rebecca and Hannah, they say siblings are mirrors of your possibilities, thank you for reflecting so brightly. Many thanks go to Tyler, Boxie and Val, for their friendship and support both then and now. And to Andrew, thank you for helping me become not only a better scientist but also a better person.
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<th>Definition</th>
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<tbody>
<tr>
<td>ACAT</td>
<td>Acyl-coenzyme A:cholesterol acyltransferase</td>
</tr>
<tr>
<td>ACBD</td>
<td>Acyl-coenzyme A binding domain containing protein</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AdR</td>
<td>Adrenodoxin Reductase</td>
</tr>
<tr>
<td>Adx</td>
<td>Adrenodoxin</td>
</tr>
<tr>
<td>AKAP</td>
<td>A kinase anchoring proteins</td>
</tr>
<tr>
<td>ANT</td>
<td>Anion Nucleotide Transporter</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy Related Proteins</td>
</tr>
<tr>
<td>Bal-A1</td>
<td>Balfiomycin A1</td>
</tr>
<tr>
<td>BN-PAGE</td>
<td>Blue-native polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CRAC</td>
<td>Cholesterol-recognition amino acid consensus</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>C27 cholesterol side chain cleavage cytochrome p450 enzyme</td>
</tr>
<tr>
<td>DBI</td>
<td>Diazepam Binding Inhibitor</td>
</tr>
<tr>
<td>DGAT2</td>
<td>Acyl CoA:diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
</tbody>
</table>
DMEM/Ham’s F-12  Dulbecco’s Modified Eagle’s medium Ham’s F-12
ER  Endoplasmic Reticulum
FBS  Fetal Bovine Serum
FRET  Förster resonance energy transfer
FSH  Follicle-stimulating hormone
GD  Geldanamycin
hCG  Human chorionic gonadotropin
HDL  High Density Lipoprotein
HMGR  HMG-CoA reductase
HSL  Hormone-sensitive lipase
Hsp  Heat Shock Protein
IMM  Inner mitochondrial membrane
LD  Lipid Droplets
LDL  Low Density Lipoprotein
LH  Lutentizing Hormone
MAM  Mitochondria associated membrane
MLN64  Metastatic lymph node 64 protein
MPTP  Mitochondria permeability transition pore
MS  Mass Spectrometry
NADP  Nicotinamide adenine dinucleotide phosphate
NB  Novobiocin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NPC</td>
<td>Niemann-Pick disease, type C 1</td>
</tr>
<tr>
<td>ODNs</td>
<td>Oligodeoxynucleotides</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>PAP7</td>
<td>PBR associated protein 7</td>
</tr>
<tr>
<td>PBR</td>
<td>Peripheral-type Benzodiazepine Receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI(3)K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RIα</td>
<td>Regulatory subunit isoforms Iα and Iβ, IIα and IIβ</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SBD</td>
<td>Sterol-binding domain</td>
</tr>
<tr>
<td>SCP-2</td>
<td>Sterol Carrier Protein-2</td>
</tr>
<tr>
<td>SR-BI</td>
<td>Scavenger Receptors Class B Type 1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic Acute Regulatory protein</td>
</tr>
<tr>
<td>START</td>
<td>Steroidogenic acute regulatory (StAR)-related lipid transfer protein</td>
</tr>
<tr>
<td>TNN</td>
<td>Triakontatetraneuropetide</td>
</tr>
</tbody>
</table>

x
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOM</td>
<td>Translocase of outer mitochondrial membrane</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratriacopeptide Repeat</td>
</tr>
<tr>
<td>TSPO</td>
<td>Translocator protein (18 kDa)</td>
</tr>
<tr>
<td>SAM</td>
<td>Sorting and assembly machinery complex</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependant anion channel</td>
</tr>
<tr>
<td>WT</td>
<td>Wortmannin</td>
</tr>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
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Introduction:

Cholesterol and Steroid Synthesis

Cholesterol is the sole precursor of steroids. Steroid synthesis is initiated at the inner mitochondrial membrane (IMM), where the cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone (1). Pregnenolone then enters the endoplasmic reticulum (ER) where further enzymatic reactions occur to produce the final steroid products. It has been shown that the translocation of cholesterol from the outer mitochondrial membrane (OMM) to the IMM is the rate-limiting step in the production of all steroids (2;3). Therefore, the ability of cholesterol to move into mitochondria to be available to CYP11A1 determines the efficiency of steroid production.

The production of steroids is regulated by trophic hormones, specifically, the adrenocorticotropic hormone (ACTH) in adrenocortical cells and luteinizing hormone (LH) in testicular Leydig and ovarian cells (2;3). The presence of these hormones activates the G protein-coupled receptors, which release the stimulatory subunit, resulting in the activation of adenylyl cyclase and a rise in intracellular cAMP (4). This increase in cAMP leads to an increase in lipid synthesis, protein synthesis, and protein phosphorylation. All these process have been shown to play a role in steroidogenesis and assist with cholesterol trafficking to the mitochondria.

Mitochondria are relatively cholesterol-poor organelles, with the majority of cholesterol located in the OMM. In the mitochondria of steroidogenic cells the pool
of cholesterol available for steroidogenesis is segregated from the structural membrane cholesterol and is bound to the cholesterol-binding domain of the translocator protein (18 kDa, TSPO), formerly called the peripheral-type benzodiazepine receptor (PBR) (5). It is from this site that cholesterol is released under hormonal stimulation to move to the matrix side of the IMM, where the cholesterol side chain cleavage enzyme CYP11A1, which will metabolize cholesterol to pregnenolone, is located. This pathway will be discussed in detail later.

As the initial translocation of cholesterol from TSPO is not sufficient to sustain the continuous production of elevated concentrations of steroids, additional free cholesterol must be moved from intracellular stores to the mitochondria. This intracellular cholesterol is known to come from three sources: i) de novo synthesis of cholesterol in the endoplasmic reticulum (ER) ii) mobilization of cholesterol in the plasma membrane with further uptake of circulating cholesterol esters from receptors found on the plasma membrane and iii) mobilization of cholesterol in lipid droplets (LD) (Fig. 1).

**Cholesterol sources in the cell**

With 65% to 80% of the total cellular cholesterol located in the plasma membrane, comprising 20% to 25% of the total lipids present, cholesterol plays a significant role in the structure and function of the plasma membrane. These interactions affect the organization of proteins and lipids in the membrane, alter the permeability of the membrane, and initiate the formation of lipid rafts (6). The
second highest concentration of cholesterol is found in the endosomal pathway, with the majority found in the endosomal to trans-Golgi compartment (6). While the majority of total cholesterol found in the cell is located in the plasma membrane, the ER contains only 1% to 2% of the total cell cholesterol (7). This gradient provides a mechanism for transport of cholesterol inside the cell from the ER to the plasma membrane and allows it to be recycled back (8).

**ER Cholesterol**

Every cell in the body is capable of producing cholesterol, with endogenous cholesterol production occurring at high rates in the liver and the brain (9). Steroidogenic organs besides the brain, such as the adrenals, testis and ovaries, are also known to synthesize cholesterol at high rates. The cholesterol synthesis pathway was initially proposed to use acetate (Fig. 1A) upon the observation that ingested deuterated acetic acid leads to the formation of deuterated acetic acid cholesterol. This led to the proposal that intermediates of isoprene units to form cholesterol (Fig 1B) (10). This model was confirmed when it was shown that two molecules of acetyl-CoA condense to form acetoacetyl-CoA, another molecule of acetyl-CoA then combines to form hydroxymethylglutaryl-CoA (HMG-CoA) a reaction mediated by HMG-CoA synthase (Fig 1C) (11;12). The reduction of HMG-CoA to mevalonate, the rate-limiting step in cholesterol synthesis, is catalyzed by the enzyme HMG-CoA reductase (HMGR) (13). HMGR is anchored to the ER, an organelle which functions as the cholesterol sensor of the cell. The ER regulates
endogenous cholesterol production primarily through the sterol regulatory element binding protein (SREBP) complex (14;15). The SREBP proteins are translocated to the Golgi upon a decrease in cholesterol and upon arrival SREBP is cleaved by two proteases. The resulting N-terminus is an active transcription factor that translocates to the nucleus. This results in an increase in the activity of cholesterol transcription genes, including HMGR (16). As HMGR is the rate-limiting enzyme in cholesterol synthesis, it is easily degraded under the high sterol conditions and can be inhibited in low levels of ATP (17). HMGR is upregulated in the presence of hormones, resulting in an increase in cholesterol production under hormonal stimulation (14). The increase of cholesterol via this pathway though has not been shown to play a primary role in steroid production (18).

After the irreversible step of mevalonate synthesis is complete, the mevalonate is then converted into two isopenoid intermediates, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (Fig 1D). Pyrophosphomevalonate decarboxylase catalyzes the formation of IPP from mevalonate requiring both NADPH and ATP. IPP can be converted into dimethylallyl pyrophosphate by isopentenyl pyrophosphate isomerase, these two compounds are then used to generate geranyl pyrophosphate combined head to tail by prenyl transferase (Fig. 1D) (12). Addition of second IPP is added in a head to tail condensation by prenyl transferase to the geranyl pyrophosphate to yield farnesyl pyrophosphate. These reactions occurring to produce farnesyl pyrophosphate from mevalonate have been
shown to occur both in the peroxisomes and the cytosol (19). The formation of squalene occurs with the head to head addition of two farnesyl pyrophosphates by squalene synthase. This generation of squalene is actually the first committed step for the synthesis of cholesterol, as previously the farnesyl pyrophosphate could be used to farnesylate proteins and form ubiquinone amongst many other cellular functions (20). The linearized squalene figure in Fig 1D shows the individual additions of carbon from the isopenoid intermediates present in Figure 1D.

From the formation squalene (represented in circular form in Fig 1E) the conversion to lanosterol proceeds via an oxygen-dependent epoxidation to give 2,3-epoxysqualene, this reaction is catalyzed by squalene exposidase. This molecule then undergoes enzymatic cyclization to yield lanosterol by 2,3-Oxidosqualene:lanosterol cyclase (OSC) (Fig 1E) (21). The activity of OSC, whose expression is also regulated by SREPBs, yields intermediates that control the liver X receptor (LXR). The activity of the LXR affects the regulation of cholesterol efflux into the cell, though which inhibition of OSC has shown to lower LDL present in the bloodstream, providing a new potential drug to suppress atherogenesis (21). The conversion of lanosterol is a nineteen step process that requires both oxygen and NADPH and release three methyl groups to produce the final product of cholesterol (Fig. 1 E) (12).

As cholesterol synthesis is tightly regulated in the ER during hormonal stimulation, cholesterol transport out of the ER is tightly controlled as well.
Cholesterol flux from the ER can occur through many pathways, including cytosolic lipid transfer proteins, through intracellular compartments or passive diffusion through contact sites. Contact sites are common between the ER and other intracellular organelles, facilitating cholesterol flux out of the ER. ER-mitochondrial contact sites have been identified in which mitochondria-associated membranes (MAM) cluster with stacks of ER (22). The cholesterol generated in the ER could potentially use this pathway for receiving steroidogenic cholesterol (Fig. 2, Pathway 1). Recently an ER protein, acyl CoA:diacylglycerol acyltransferase 2 (DGAT2) was found associated with both LD and the mitochondria while still present in the ER. As DGAT2 functions in the final stage of triglycerol synthesis it is possible that this is a pathway for lipid transfer (23). Currently it is unknown whether an interaction between the ER and mitochondria occurs in steroidogenic cells; therefore further studies will be necessary to investigate the presence and function of such an interaction in steroidogenesis.

**Plasma Membrane**

Cholesterol is primarily stored in the plasma membrane. Upon hormonal stimulation there is increased cholesterol absorption through the plasma membrane. When cholesterol is imported into the cell via the plasma membrane it greatly increases the cholesterol content stored elsewhere in the cell. This was observed when an increase of 50% in cellular cholesterol absorbed via the plasma membrane resulted in a 10-fold increase in ER cholesterol (24). Currently, there are two known
pathways for this cholesterol absorption and import into the cell: a non-selective endocytic pathway and a “selective” absorption pathway. In the non-selective pathway LDL molecules are specifically bound and internalized via the LDL receptors. Once the receptor has been internalized it fuses with the endosomal pathway for distribution of the lipoproteins (Fig. 1, Pathway 2). The “selective” pathway uses scavenger receptors class B type I (SR-BI) located at the plasma membrane to bind both LDL and HDL. Through local binding mechanisms the cholesterol present in the lipoproteins is transferred directly to the cell membrane without absorption of the lipoprotein particles (25) (Fig. 1, Pathway 3). Further analysis of these two pathways has shown that adrenal steroidogenesis is dependent primarily on HDL cholesterol absorbed from the plasma membrane, primarily via the SR-BI pathway (26).

The non-selective vesicular pathway is initiated primarily by LDL particles binding to the LDL receptor, followed by the endocytosis and budding of clathrin-coated pits into the cytoplasm (27) (Fig 1, Pathway 2). These vesicles fuse with early endosomes, releasing their clathrin coats and allowing the LDL receptors to cycle back to the membrane. This endosomal fusion and trafficking occur through interactions with microtubules which are controlled through Rabs (28). Rabs are small GTPases that regulate membrane traffic through binding at their active site, currently there have been more then 60 proteins identified in mammalian cells (29;30). The early endosomes bind to recycling endosomes coordinated by Rab5,
and then to late endosomes via Rab 7, to further distribute cholesterol throughout the cell (29;31). The endosomes also undergo a decrease in pH from 7.4 at the plasma membrane to 5.5 - 6 at the late endosome (32). This decrease in pH helps further dissolve the absorbed lipoprotein and prepares the late endosomes to fuse with the lysosomes.

It has been shown that the LDL receptor is not necessary for acute adrenal steroidogenesis, suggesting that cholesterol absorbed via this pathway is not necessarily used for steroidogenesis (33). However, in FSH- or FSH plus androstenedione-treated granulosa cells the rate of LDL receptor absorption increases while the time needed for the LDL to reach the lysosome decreases compared to non-hormone-treated cells suggesting this pathway is used for steroidogenesis (34). As endosomes contain a large percentage of the cytosolic cholesterol present in the cell; they can function in the trafficking of intracellular cholesterol to the mitochondria without first absorbing cholesterol from the plasma membrane. This pathway could occur specifically through the cholesterol rich late endosomes, shown to fuse with the lysosomes and the Golgi apparatus and transiently interacting with the mitochondria, thus allowing for multiple sources of cholesterol to be available to the mitochondria. Endosomal trafficking in the cell has also been shown to be altered by cholesterol concentrations, specifically via Rab7, suggesting a mechanism by which trafficking to the mitochondria could be regulated (35).
The second pathway identified for cholesterol absorption and trafficking in steroidogenic cells occurs through the action of the SR-BI receptor (36) (Fig 1, Pathway 3). The SR-BI receptor is found in many tissues such as intestines, macrophages, and endothelial cells, though it is expressed in highest concentrations in steroidogenic tissues such as the adrenals, ovary, and testis (25). Unlike the LDL receptor in which the apoprotein is absorbed, the SR-BI receptor forms a non-aqueous channel that allows a large influx of cholesterol directly into the plasma membrane (25). This non-aqueous channel has also been shown to be regulated in the intestines through other proteins found in the plasma membrane, such as CD36 and other proteins yet to be identified (37). Suggesting this pathway might require a complex, multiprotein interaction to regulate cholesterol absorption into the plasma membrane. Because SR-BI absorbs free cholesterol from HDL and stores it in the plasma membrane, this cholesterol can move spontaneously between bilayers and membranes in the cell without the assistance of any proteins. This process is slow and therefore not suggested as a pathway involved in the acute stimulation of steroidogenesis (38). The esterified cholesterol absorbed from the SR-BI receptor must be converted to free cholesterol before it can be used for steroidogenesis via a cholesterol ester hydrolase (39). In steroidogenic cells cholesterol ester hydrolysis is performed through hormone-sensitive lipase (HSL) (40). HSL becomes activated when phosphorylated by cAMP; inhibition of HSL results in decreased steroidogenesis in the adrenals and inhibits sperm production in the testis (41-43).
The cholesterol absorbed via the plasma membrane has been shown to be hydrolyzed rapidly, presumably close to the plasma membrane, to form free cholesterol. Once the cholesterol esters have undergone hydrolysis, the HSL can interact with various cholesterol-binding proteins to direct the cholesterol to the OMM for steroidogenesis (44). This pathway involving cholesterol-binding proteins will be discussed later in detail.

Lipid Droplets

Lipid droplets (LD) are bounded by a phospholipid membrane and function as a repository of cholesterol esters and triglycerides in the cell. It has been proposed that LD form from the ER when excess neutral lipids bud off, although there is no direct evidence to support this model (45). The cholesterol esters found in the LD are the products of the ER enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT), which becomes active in the presence of high levels of cholesterol. This enzyme attaches an ester to the free cholesterol found in the ER, increasing the cholesterol ester content present in the cell (46). Because lipids in the LD can be used for various biological activities their size fluctuates depending upon the cell’s activity. In steroidogenic cells, LDs are small to increase the surface area for lipid retrieval (45).

The cholesterol esters present in the LD are also converted to free cholesterol in the same manner as cholesterol absorbed via the SR-BI receptor, i.e., through HSL. Transfer of steroidogenic cholesterol from intracellular organelles to the
mitochondria is thought to occur through cholesterol-binding proteins found in the cytosol (Fig 1, Pathway 4) (47). Other mechanisms for lipid transport from the LD have been demonstrated. Rab5, which localizes to early endosomes, has been shown to interact with LD, suggesting a mechanism for cholesterol transfer from the LD to the endosome and vice versa, allowing for an increase in cholesterol in the endosomal pathway (48). Rab18 also associates with LD, regulating the contact between the lipid droplet and the ER, which controls the flux of cholesterol during lipolysis (49). Since LDs play an important role in regulating intracellular cholesterol through storage, trafficking, and esterification, further studies are needed to determine the endosome/LD interaction which would allow for transfer or fusion of the cholesterol from the LDs into the early endosomal pathway for steroidogenesis.

**Targeting cholesterol to the mitochondria**

The primary pathway for targeting cholesterol to the mitochondria has not been definitively identified. Two pathways have been proposed: (i) the non-vesicular pathway involving cholesterol-binding proteins transferring cholesterol through the cytosol to the mitochondria, and (ii) a vesicular pathway characterized by an increase in the fusion of vesicular membranes, such as endosomes and lysosomes, which results in an increase in cholesterol targeted to the mitochondria (50). An overlap between these two pathways is highly likely as cholesterol binding proteins have been found on endosomes; this suggests that an increase in cholesterol
targeting to endosomes could also have a direct effect on cholesterol-binding proteins which target cholesterol for transfer to the mitochondria.

**Sterol carrier protein-2**

Sterol carrier protein-2 (SCP-2) was one of the initial cholesterol binding proteins identified; SCP-2 was shown to play a role in the intracellular transfer of cholesterol, including from the lysosomal to mitochondrial membranes (51;52). SCP-2 is found in tissues involved in cholesterol trafficking and oxidation, such as the liver, intestines, adrenal, testis, and ovary, suggesting it could play a role in steroidogenesis (53). Further studies showed SCP-2 increases cholesterol uptake and transport throughout the cell while inhibiting the efflux of cholesterol from the cell through the HDL receptor (54;55). Because no alteration in steroidogenesis was observed in SCP-2 knock-out mice, it has been assumed that SCP-2 does not play a primary role in steroidogenesis in vivo, though other interactions are still possible (56). This focused attention on other identified cholesterol binding/transfer proteins.

**START domain proteins**

The steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain is an amino acid motif that has been proposed to play a role in cholesterol and lipid binding (57) (Fig. 3A). The motif first was identified in the StAR protein, discussed below, which has been shown to play a role in cholesterol transport to the mitochondria for steroidogenesis. The 210-amino-acid sequence forms a beta sheet core surrounded by two alpha helices, resulting in a hydrophobic channel that can
hold a sterol molecule capped by the C-terminal alpha helix (47). The structure becomes stable upon binding of the cholesterol molecule, which has been shown to bind at a 1:1 ratio (58).

MLN64, the second identified StART domain protein, was shown to be upregulated in breast and ovarian cancers and found to have a C-terminal domain with 37% amino acid identity and 50% amino acid similarity to the C-terminal domain of StAR (59;60). Further studies confirmed that this was a START domain that could bind cholesterol and transfer free cholesterol from sterol-rich vesicles to acceptor membranes. MLN64 was found to be localized primarily on late endosomes, integrating at the plasma membrane and functioning in the vesicular trafficking of LDL cholesterol (61) (Fig 1). When the START domain was removed from MLN64, cholesterol accumulated in the lysosomes and altered late endosome trafficking (62). In COS-F2 cells, this accumulation of cholesterol suppressed steroidogenesis, presumably by limiting the efflux of free cholesterol from the late endosomes and lysosomes to the mitochondria.

MLN64 was also found to be associated with Niemann-Pick type C disease protein 1 (NPC1) in the late endosomes (63). Niemann-Pick type C disease (NPC) is a disorder characterized by the accumulation of LDL-derived unesterified cholesterol in the late endosomes and lysosomes caused by mutations in either NPC1 or NPC2 (64). NPC1 gene expression has been shown to be responsive to cAMP and essential for the normal development of the adrenals (65). NPC1 is found bound to late
endosomal membranes while NPC2 is found inside the late endosomes, early lysosomes, and in the cytosol. The presence of NPC2 accelerates the transfer of free cholesterol from late endosomes to lysosomes and cholesterol efflux to the plasma membrane (66;67). NPC1 is necessary for cholesterol efflux from the late lysosome for use in the cell (32). When steroidogenic human granulosa-lutein cells deficient in NPC1 protein were used for studying cholesterol trafficking, steroidogenesis levels decreased to levels seen with LDL-deficient media (68). This observation suggests that the NPC1/late endosome pathway is used primarily for LDL-derived steroidogenesis. As NPC1 and NPC2 function in the efflux of cholesterol from the endosome, this pathway could also interact with MLN-64 for steroidogenic purposes.

Steroidogenic acute regulatory (StAR) protein was first identified due to its rapid phosphorylation and protein expression soon after the addition of hormones and cAMP in steroidogenic cells (69-71). Expression was confirmed in the adrenal cortex, testis, and ovary and later in the brain and placenta, suggesting a connection between StAR protein expression and steroid production (72;73). Transfection of StAR expression vectors in both mouse Leydig MA-10 cells and COS F2 cells, which contain the components of CYP11A1, was found to increase steroidogenesis (74;75).

There have been many models proposed for the function of StAR in steroidogenesis (76). It was suggested early that StAR assists with the transfer of cholesterol to the mitochondria, although no clear mechanism was identified.
Because StAR contains an N-terminal mitochondrial-targeting sequence, which is cleaved from its active molecular weight of 37 kDa to its inactive weight of 30 kDa upon import into the mitochondrion, it was proposed that this targeting sequence could assist with the formation of a mitochondrial “contact site”. This would be accomplished by the interaction of StAR with mitochondrial protein import complexes found on both the OMM and IMM and allow the N-terminus to form a linker connecting the membranes. Cholesterol would then be able to flow from the OMM to the IMM and interact with CYP11A1. However, removal of the N-terminus targeting sequence in the construct N-62 StAR and transfection into steroidogenic cells had no effect on steroidogenesis, suggesting this is not the mechanism by which StAR facilitates cholesterol transfer (77). It was later observed that N-62 StAR was able to insert cholesterol into cytosolic membranes other than the mitochondria, suggesting that the primary function of the N-terminal sequence is not to form mitochondrial contact sites but to limit StAR’s cholesterol targeting abilities solely to the OMM (78).

The effect of StAR on mitochondrial steroidogenesis was further confirmed by fusing mitochondrial translocases to the N-62 StAR construct. Fusion of N-62 StAR with Tim9, a mitochondrial inner membrane space protein, or Tim40, an IMM protein, resulted in no increase in steroidogenesis while fusion of N-62 StAR with Tom20, an OMM protein, resulted in maximal production of steroids (79). This observation demonstrated that StAR’s site of action was confined to the OMM and
that, once imported, StAR does not stimulate steroidogenesis. Because StAR interacts only transiently with the OMM before being imported, the amount of time StAR spends at the OMM would be able to alter the rate of steroid production. This was shown when a StAR/StAR construct, which is imported at a slower rate than wild-type StAR, was shown to increase pregnenolone production over both wild-type and N-62 StAR construct levels in COS-F2 cells (79). This finding shows that StAR functions primarily at the OMM, possibly activating a pathway of cholesterol transport for steroidogenesis.

Another proposed model built to explain StAR’s activity suggested that it could function as an intermembrane cholesterol shuttle, moving cholesterol from the outer to the inner mitochondria membrane one molecule at a time during StAR’s import (58). Tsujishita and Hurley proposed that during StAR’s import into the mitochondria several molecules of cholesterol could be transferred through the import of one StAR molecule through transient openings. Several issues were raised with the model, including the lack of understanding of how StAR could both bind cholesterol and then release it in the IMM space. It was also uncertain how StAR would reside in the IMM space as it does not have a mitochondria targeting sequence for the inner mitochondria membrane space.

One of the more current proposed models is the “molten globule” model identified through the studying of the StAR’s C-terminus. Removal of the last 10 C-terminal amino acids resulted in decreased steroidogenesis, while removal of the 28
C-terminal amino acids resulted in a biologically inactive protein, suggesting that StAR’s mode of action was occurring at the C-terminus. Further studies showed that the C-terminus forms a sterol-binding domain (SBD) with which a cholesterol molecule is proposed to interact. The SBD forms a pocket that prevents the release of the bound cholesterol molecule, which can occur only following a conformational change. This is proposed to occur through the “molten globule” configuration in which tertiary structures are removed, allowing the remaining secondary structures to undergo a conformational shift and allow the cholesterol molecule to enter the mitochondria. The molten globule model has been proposed to occur through the protonation of the C-terminus at the OMM. Since binding of cholesterol to the SBD in the StAR protein has not been definitively demonstrated, it is still not clear if this is the mechanism by which cholesterol is transferred to the OMM. It should be noted that the cholesterol-binding activity of StAR is independent of its activity at the OMM, because StAR mutant R182L can still function in the binding and transfer of cholesterol in isolated liposomes, although this results in an increase in cholesterol present in the cell (80). This suggests that cholesterol binding is necessary but not sufficient for StAR’s function on the OMM.

Further homology modeling and biophysical studies recently indicated the existence of a two-state model (81). The first and open state of the model proposes that the C-terminal alpha-helix 4 of StAR, acting primarily as a gating mechanism to the cholesterol binding site, undergoes partial unfolding allowing cholesterol to bind.
This resulting cholesterol bound state, in theory, would lead to the stabilization and the refolding of alpha-helix 4; resulting in a well-defined tertiary structure (81). This stable tertiary structure could be necessary for protein-protein interactions formed for cholesterol transfer at the surface of the mitochondria, suggesting that both StAR conformation and cholesterol binding are necessary for its proper steroidogenic activity.

These models of StAR’s function have been able to provide many descriptions of cholesterol transport to the mitochondria which can relate to the function of START proteins. As six other START-domain families have been identified, such as StarD4, StarD5, and StarD6, the understanding of how the START domains function can be applied to these proteins as well (82). This can have far reaching effects, as compared to StAR and MLN64; StarD4, StarD5, and StarD6 lack an organelle-targeting sequence and therefore are thought to be cytoplasmic. StarD4 and StarD5 are widely expressed, while StarD6 is found primarily in the testis (57). This ability of the START domain to target and transport cholesterol from multiple sources, including the plasma membrane, ER, and endosomes, could ensure a large source of cholesterol for steroidogenesis.

**Importing Cholesterol into the Mitochondria**

Cholesterol successfully imported via the plasma membrane or accessed in LDs and transported to the OMM remains segregated in the OMM until translocation
to the IMM. This, the rate-limiting step in steroidogenesis, has been suggested to occur primarily through TSPO.

**TSPO and cholesterol**

TSPO was first identified by the presence of radiolabeled diazepam binding in the kidney (83) and it was later found to be present in most tissues of the body (84). It was proposed that TSPO plays a role in steroidogenesis when ligand-binding studies revealed increased expression of TSPO in steroidogenic tissues and subcellular localization studies indicated that it was primarily localized to the OMM (84-87).

TSPO was recognized using a benzodiazepine drug ligand specific for the GABA<sub>A</sub> receptor in the central nervous system; however, subsequent identification of TSPO-specific ligands allowed it to be differentiated from the GABA<sub>A</sub> receptor. This was accomplished by use of the isoquinoline carboxamide PK 11195, which binds with nanomolar affinity to TSPO but has no affinity for the GABA<sub>A</sub> receptor (88). Many endogenous TSPO ligands exist in the cell, with porphyrins being able to bind TSPO with high nanomolar affinity (89). The diazepam binding inhibitor (DBI) is another endogenous ligand. This 10 kDa protein, which also binds the GABA<sub>A</sub> receptor with low affinity, is expressed in many tissues but is primarily expressed in steroidogenic tissues where it is localized in the cytosol in contact with the OMM (90). Naturally processed peptides of DBI, octadecaneuropeptide (ODN, DBI<sub>33-50</sub>) and triakontatetraneuropeptide (TNN, DBI<sub>17-50</sub>), expressed in a hormone-dependent manner, were functional in binding TSPO in the brain, adrenal, and testis.
DBI and its peptides stimulated mitochondrial pregnenolone formation. When DBI expression was suppressed in the presence of antisense oligonucleotides MA-10 Leydig cells failed to respond to hormonal stimulation and steroid production was inhibited.

Early experiments in multiple steroidogenic cell systems showed that pregnenolone production was stimulated upon exposure of the cells to TSPO ligands (92;93). When these experiments were repeated in isolated mitochondria incubated with TSPO ligands a similar increase in pregnenolone was observed (85;93). This increase was not seen in mitoplasts, mitochondria devoid of their OMM and therefore deficient in TSPO. To determine the effect of TSPO ligands on the mitochondria, cholesterol content in the OMM and the IMM was measured both before and after TSPO ligand treatment (94). This study revealed that ligand binding to TSPO induced the translocation of cholesterol from the OMM to the IMM and confirmed that TSPO participates in the binding and release of cholesterol at the OMM, an initial step in the production of steroids.

To further confirm TSPO’s role in cholesterol binding and translocation a bacterial expression system was devised because bacteria contain no endogenous cholesterol. *E. coli* were transformed with an inducible mouse cDNA TSPO vector, resulting in fully expressed TSPO. Ligand-binding experiments were performed to verify that the bacterial TSPO possessed the same pharmacological binding properties as native TSPO; this was confirmed when bacterial TSPO bound both
cholesterol and PK 11195 with nanomolar affinities (95). When bacterial TSPO was incubated with radiolabeled steroids, time- and temperature-dependent uptake of cholesterol was seen in the protoplasts although no uptake of other steroids was seen. When the bacterial cholesterol-loaded membranes were treated with PK 11195, the cholesterol was released (95). These findings confirm that TSPO functions as a cholesterol translocator and suggest that TSPO might further function as a cholesterol sink, holding cholesterol until it is released by the binding of a ligand.

To identify the basis of the interaction of TSPO with cholesterol, molecular modeling and site-directed mutagenesis were used to identify potential binding sites. Previous studies had shown that TSPO spans the OMM in five alpha helices, composed of approximately 21 amino acids each. The 3-D models produced suggested the five alpha helixes come together to form a channel with a hydrophilic but uncharged interior surface (95) (Fig. 3B). It was shown that the interior of the channel could bind a cholesterol molecule that had not been significantly modified, suggesting that TSPO could function as a transporter of cholesterol to the IMM. To identify the cholesterol-binding domain several deletion constructs were generated. A region on the C-terminus (Δ153-169) was identified as necessary for cholesterol binding by virtue of the mutant’s reduced ability to take up cholesterol, although PK 11195 binding was unaltered (96). Further site-directed mutagenesis experiments identified the specific amino acids necessary for cholesterol binding, yielding a CRAC (cholesterol-recognition amino acid consensus) domain (Fig. 3C & D).
CRAC domain showed the high nanomolar affinity for cholesterol that had been observed in other proteins interacting with cholesterol (96). These data suggest that the C-terminus of TSPO plays an important role in the uptake and translocation of cholesterol into the IMM.

To confirm the role of TSPO in ligand-binding and cholesterol translocation, a peptide antagonist was developed using a random seven-mer peptide library attached to an HIV-TAT domain (97). The TAT domain allows receptor-independent entry of the protein or peptide attached to the sequence (98). The random seven-mer peptides attached to the TAT domain were incubated with MA-10 Leydig cells and peptides eluted with ligand Ro5-4864. It was shown that the domain STXXXXP, specifically STPHSTP, competed with the highest efficiency for the ligand-binding domain (99). This was further confirmed when hormone-induced steroidogenesis was shown to be inhibited by this peptide in a dose-dependent fashion. The CRAC domain was then also fused to the TAT domain, allowing entry into MA-10 cells in a dose-dependent manner (96). This was shown to inhibit steroidogenesis through a dominate-negative effect by altering the translocation of cholesterol from the mitochondria to the TAT-CRAC domain. These data confirmed the importance of the C-terminus in the binding and translocation of cholesterol. In both cases the production of steroids from 22R-hydroxycholesterol was not altered, demonstrating that the peptide affected only TSPO and its ability to bind cholesterol and endogenous ligands(96;99).
From these experiments it was shown that TSPO is a high-affinity cholesterol- and drug ligand-binding protein. It functions in the translocation of cholesterol from the OMM to the IMM in the presence of its ligands. It should be mentioned that due to the important role of cholesterol in mammalian cells and the diverse localization of TSPO in many tissues, TSPO may play a more extensive role in the cell, participating in targeting cholesterol to mitochondria for membrane biogenesis and also cholesterol transport in the cell. This idea gained further support when treatment of both steroidogenic and non-steroidogenic cells with TSPO ligands resulted in a redistribution of cholesterol from the plasma membrane to LD (100), suggesting a possible role for TSPO in the intracellular regulation and trafficking of cholesterol, independent of cell type.

The next step was to determine if TSPO was solely responsible or if other proteins could be assisting with this translocation of cholesterol in the mitochondria. To do this the R2C rat Leydig cell line, derived from rat Leydig tumors and shown to constitutively produce steroids (101), was used. The TSPO gene was disrupted by homologous recombination, resulting in a dramatic decrease in steroid production to 10% of control values (102). However, when 22R-hydroxycholesterol, a hydrophilic CYP11A1 substrate that can pass directly into the IMM, was added the levels of steroid production returned to normal (102). The role of TSPO in steroidogenesis was further verified when a TSPO knock-out mouse model proved to be embryonic
lethal (103), demonstrating that TSPO is not only necessary for steroidogenesis but it also plays a critical role in early embryonic development.

Hormonal stimulation in steroidogenic cells initiates the transfer of cholesterol from the OMM to the IMM. As TSPO is suggested to play a role in this process, the effect of hormones on TSPO activity was examined. Upon the addition of the gonadotropin hCG in MA-10 Leydig cells, TSPO was shown to cluster in groups of four to six molecules (104;105). This clustering results in increased ligand binding, cholesterol dispersal into the IMM, and steroid production and has been shown to increase the formation of contact sites. The clustering of TSPO caused by hCG can be inhibited by the addition of a cAMP-dependent protein kinase (PKA) inhibitor, suggesting that localization and clustering of TSPO is a cAMP-inducible event (106). Because antibodies against TSPO recognize immunoreactive proteins of molecular weight greater than 18 kDa, it has been suggested these were polymers of TSPO formed through the hormonally induced clustering. The clusters of TSPO have been shown to be due to the formation of permanent dityrosine bonds (107). Bond formation is achieved through the generation of reactive oxygen species (ROS) triggered by the presence of hormones in Leydig cells. This has been further confirmed to follow the pathway of induction of cAMP-induced and PKA-dependent ROS formation via the mitochondrial respiration complex I (108).

To better understand the role of drug ligands in cholesterol transport in the mitochondria NMR analyses were performed. The results showed that the alpha
helical structure of TSPO was present in the monomer form, while the overall
tertiary structure was somewhat less structured (109). The presence of the drug
ligand PK 11195 stabilized TSPO, providing a more stable environment for the
translocation of cholesterol to the IMM.

**Interactions of TSPO and mitochondrial proteins:**

Once cholesterol has been bound to TSPO it is committed to use in
steroidogenesis. Because TSPO is located primarily at mitochondrial contact sites
(110), it has been suggested that TSPO does not function alone in the OMM. Native
TSPO in digitonin solubilized mitochondrial extracts elutes on gel filtration column
chromatography in a digitonin containing buffer as a 200- to 240 kDa complex
(unpublished results) while cross-linked TSPO solubilized with digitonin elutes at
170 to 210 kDa (111;112). Studies identifying proteins in these complexes showed
TSPO to be eluting at 18, 36, and 54 kDa, presumably representing the monomer and
polymers of TSPO induced by hCG. Other proteins eluted were identified as
voltage-dependent anion channel (VDAC), adenine nucleotide transporter (ANT),
and unidentified proteins at 60 kDa (Fig 4A) (113;114).

VDAC is an OMM channel-forming protein that regulates the passage of ions
and small molecules through the OMM. This function determines membrane
potential, thus assisting with the regulation of apoptosis and cell metabolism (115).
VDAC’s interaction with ANT forms the mitochondrial permeability transition pore
(MPTP), which is located primarily at mitochondrial contact sites and regulates the
cell’s response to apoptotic events. ANT’s role in the MPTP has been shown not to be essential though it is believed that it might function in a regulatory manner (116). Its interactions with TSPO are currently unknown though it has recently been shown that ANT can also bind an identified TSPO ligand, protoporphyrin IX, and transport it into the mitochondrial matrix (117). It has been suggested that interactions of TSPO, VDAC, and ANT might modulate the cell’s response to apoptotic signals. Because TSPO has been shown to be involved in ROS production, it has also been suggested that TSPO might function in the apoptotic response (118). It has also been suggested that MPTP can alter steroidogenic rates as it is known that Leydig cell mitochondria need to be fully functional for steroidogenesis and that the opening and closing of the MPTP alters the cells’ ability to produce steroids (unpublished data). As the number of contact sites can be modulated (increased) by hormone treatment; which in theory, could increase cholesterol transport between the OMM and the IMM, the permeability of the mitochondria could also affect steroidogenesis, regulated in part by TSPO (119).

Modeling has shown that VDAC binds cholesterol and it has been independently demonstrated that VDAC influences cholesterol distribution in the mitochondria (114;120;121). It is also known that PK 11195 affects TSPO tertiary structure by stabilizing the protein in the cell(109). TSPO’s loss of flexibility as a result of binding to VDAC could translate into a more rigid VDAC, altering the respiratory state and possibly cholesterol distribution in the mitochondria as well.
TSPO and cytosolic cholesterol import

TSPO and StAR

Steroidogenic cholesterol is targeted to the mitochondria though proteins containing the StART domain - StAR and MLN64 - as mentioned earlier. For this cholesterol to be used effectively for steroidogenesis it must interact with TSPO. Because TSPO and StAR have been shown to interact by FRET (122), but not BRET (123) analysis, this might be a pathway through which cholesterol can be transferred.

To determine how TSPO and StAR interact at the OMM, antisense oligodeoxynucleotides (ODNs) were used to reduce expression of the two proteins. When StAR expression was reduced, hCG-stimulated MA-10 Leydig cells stopped producing progesterone after 20 minutes, while in TSPO-depleted cells steroidogenesis was inhibited after ten minutes(124). Together these results show that both TSPO and StAR function in steroidogenesis; the difference in time of arrest of steroidogenesis was attributed to the presence of cholesterol on the OMM available to be used for steroidogenesis. Since we were unable to demonstrate a direct physical StAR/TSPO interaction we searched for a functional one. Thus, StAR expression was examined in TSPO-depleted cells. It was shown that StAR was not processed from the 37 kDa cytosolic protein to the mature intramitochondrial 30 kDa protein that is normally seen under hormonal stimulation (124). This was further confirmed when a peptide antagonist shown to bind to the
cholesterol-binding domain of TSPO also inhibited the intramitochondrial formation of the 30 kDa StAR (99;124). Based on these results it was suggested that TSPO plays a direct role in the import of StAR into the IMM and that StAR is dependent upon TSPO for its activity.

To test this hypothesis, TSPO-depleted mitochondria were transfected with a Tom/StAR construct that would be targeted to and imported into the OMM. Previously it had been shown that this construct increased progesterone production two-fold in MA-10 cells; however, no effect was seen on steroid production in TSPO-depleted mitochondria (79;124). TSPO was then reincorporated into the isolated mitochondria, restoring the ability of the mitochondria to produce pregnenolone. This effect was seen with both Tom/StAR and StAR constructs. Based on separate analysis and reintroduction of these two proteins into the cell system, it was proposed that StAR’s primary function is in the hormone-induced transport of cholesterol to the OMM while TSPO regulates the translocation of cholesterol into the IMM.

Acute stimulation of steroidogenesis in vivo results in measurable hormone production within minutes. However, in vitro this effect is not observed until 10 - 20 minutes, which is interesting because StAR protein synthesis shows a lag of 20 - 30 min after hormone stimulation (125). As previously mentioned, steroidogenic cholesterol can be found bound to TSPO in the OMM, suggesting that this time frame would deplete TSPO of cholesterol and allow StAR to then replenish
cholesterol concentrations. Limiting StAR protein expression and, therefore, its activity in the cell, would allow for cholesterol to be specifically targeted to the mitochondria for steroidogenesis and inhibit excess cholesterol transport. This delayed protein expression also confirms that StAR does not act alone on the OMM in steroidogenesis.

StAR has been shown to cycle sufficiently rapidly to transfer 400 molecules of cholesterol per minute into adrenal cells (126). However, experimental observations have demonstrated that the stoichiometry of cholesterol transfer is 1.82 molecules of cholesterol per minute in isolated mitochondria (127), suggesting that further modification in the cell is needed for maximal StAR activity. One proposed mechanism for this modification is cholesterol binding to the SBD, which has been shown to be necessary for StAR’s function (128). StAR binds cholesterol with an affinity of 32 nM (129), although binding at an affinity of 95 µM has recently been reported (130). Because StAR rapidly shuttles cholesterol through the OMM, high-affinity binding of cholesterol to StAR would not favor this transfer. It is also important to note that this affinity is substantially lower than the affinity of 5 nM of TSPO for cholesterol (131), suggesting a possible mechanism by which TSPO removes cholesterol from StAR when it is in close proximity to the OMM.

**TSPO and PAP7**

To determine if other proteins are necessary for importing cholesterol into the mitochondria a yeast-two hybrid screen was performed with TSPO as the bait. This
approach demonstrated the association of several PBR-associated proteins (PAPs) with TSPO, with PAP7 demonstrating the most compelling interaction (132). PAP7 was found to have an expression pattern similar to TSPO and was localized to the Golgi and mitochondria. Interestingly, in another yeast-two hybrid screen with the regulatory subunit R1α of PKA as the bait, PAP7 was also identified (132). These data provided useful insight into the function of PAP7 because PKA phosphorylates proteins in a hormone-specific manner through the activation of cAMP. When cAMP levels rise the proteins bind to the two regulatory subunits of PKA, RI and RII and their isoforms (Iα and Iβ, IIα and IIβ), which release the two catalytic subunits; these are then able to phosphorylate specific serine and threonine residues, activating select proteins (Fig. 4 B). It was then confirmed that PAP7 binds both TSPO and PKA-R1α in vitro in MA-10 mouse Leydig cells (132). Overexpression of PAP7 was shown to stimulate progesterone production in MA-10 cells and transfection of the TSPO- and PKA-R1α-binding domain of PAP7 (a.a. 228 - 445) significantly inhibits steroidogenesis (132;133). These results further confirm PAP7’s role in steroidogenesis via interaction with TSPO and PKA-R1α. In these studies we identified an acyl-coA binding motif in PAP7, similar to the one identified in DBI (133). More recently, DBI and PAP7 were renamed acyl-coenzyme A binding domain containing 1 (ACBD1) and 3 (ACBD3) proteins, respectively (Fan, Liu, Culty and Papadopoulos, manuscript submitted).
Since PAP7 is known to bind PKA-RIα, it was suggested that PAP7 functions as an A Kinase Anchoring Protein (AKAP). AKAPs are a family of proteins known to recruit the PKA holoenzyme into proximity to its substrate, confining its activity (134). In this process, PAP7 is presumed to bring PKA-RIα into closer proximity to the proteins mediating cholesterol transport and thus steroidogenesis, playing a role in regulating their activity via phosphorylation. This mechanism would allow the signaling mechanism for steroidogenesis to be localized to certain areas on the mitochondria, limiting the concentration of protein needed for maximal stimulation. Thus, as the concentration of trophic hormone needed to stimulate maximal cAMP production is about 15 times higher than that needed to maximally stimulate testosterone secretion (135) such a mechanism would allow maximal steroid formation in the presence of submaximal cAMP accumulation. Targeting of PKA-RIα close to mitochondrial TSPO would result in the localization and amplification of its ability to phosphorylate proteins involved in steroidogenesis.

It is known that cloned rat, bovine, and murine TSPO are phosphorylated in the C-terminal domain, although a phosphorylation site has not been identified in human TSPO (136). StAR becomes rapidly phosphorylated upon the addition of trophic hormones in all species investigated to date (137). Based on these observations it was proposed that PAP7 anchors PKA-RIα, facilitating the phosphorylation of StAR and possibly TSPO, in a cAMP-dependent manner (Fig
4B). This mechanism would allow the activity of StAR to be regulated and activated by proteins localized to the OMM, controlled by proteins known to anchor to TSPO.

To determine if the proteins under investigation interact at the OMM, COS-F2-130 and MA-10 cells were transfected with TSPO, StAR, PAP7, and PKA-RIα. The transfection of the four proteins together in the non-steroidogenic cells induced an increase in steroid production greater than that induced by each individual protein alone, suggesting that these proteins form a complex that performs a necessary role in steroidogenesis. Hormonal stimulation of non-transfected Leydig cells induced a greater increase in steroid synthesis, suggesting that the complex of proteins is still dependent upon stimulation through cAMP and not on the amount of proteins present. The interactions between TSPO, StAR, and PKA-RIα were subsequently analyzed by microscopy. These studies indicated that, upon hormonal stimulation, PAP7 translocated from the Golgi to the mitochondria (138) and StAR translocates to the mitochondria, where PKA-RIα and PAP7 colocalize with TSPO (138). These studies indicate that a protein complex is formed at the mitochondria to assist with the translocation of cholesterol into the mitochondria.
Aims:

Many advances have been made in identifying protein-protein interactions necessary for steroidogenesis. From the initial stages of cholesterol import into the plasma membrane, through lipoprotein binding to receptors, to the actual translocation of cholesterol into the IMM for pregnenolone production, each step has been shown to be tightly controlled and regulated through these interactions. Despite progress in understanding the cellular and molecular mechanisms underlying the hormonal regulation of cholesterol transport into mitochondria and, thus, of steroidogenesis, there are several issues that remain to be addressed. The primary issue, and one this thesis will address, is the molecular mechanisms necessary to regulate cholesterol trafficking to the mitochondria for steroidogenesis. This thesis addresses the hypothesis that protein-protein interactions in distinct cellular compartments dictate the flux of cholesterol in steroidogenic cells. This hypothesis will be addressed through the completion of three aims. The first aim addresses how a series of proteins, TSPO, StAR, PKA-RIα and PAP7, all interact and form a complex at the OMM to promote cholesterol transport. As cholesterol is dependant on TSPO for delivery to the IMM, the second aim proposes the import of TSPO into the OMM is regulated by protein-protein interactions as well, which has implications for steroidogenesis. The third aim proposes that in addition to the TSPO-StAR-PKA-R1α-PAP7 complex formed on the OMM, protein-protein complexes occurring in the mitochondria assist with the transfer of cholesterol from the OMM to the IMM and its conversion to pregnenolone.
CHAPTER 1:
Identification of Protein-Protein Interactions that Mediate Mitochondrial Cholesterol Transport and Steroid Biosynthesis

The work herein has been published in Jun Liu, Malena B. Rone, and Vassilios Papadopoulos. Protein-Protein Interactions Mediate Mitochondrial Cholesterol Transport and Steroid Biosynthesis. *Journal of Biological Chemistry* 2006; 281: 38879-38893
Abstract:

Transfer of cholesterol to the inner mitochondrial membrane is the rate limiting step in the production of steroids. As mitochondria are cholesterol poor organelles, the delivery of supplemental cholesterol to the mitochondria is a necessary and essential element of acute steroidogenesis. In the following study, we examine the protein-protein interactions that occur after hormonal stimulation, confirming the formation of a macromolecule signaling complex. This complex consists of the mitochondrial translocator protein (TSPO, 18 kDa), Golgi protein PAP7, the regulatory subunit R1α of cAMP- dependant protein kinase A (PKA-R1α) and the Steroidogenic Acute Regulatory (StAR) protein. This macromolecule signaling complex was also shown to contain 7-azi-5α-cholestan-3β-ol (photo-cholesterol) when it is incubated in the media. This demonstrates that the protein complex that forms on the outer mitochondria membrane contains cholesterol and a hormone dependant protein, StAR. From these observations, we conclude that this protein complex formation is necessary for steroidogenesis, playing a role in targeting cholesterol to the mitochondria.
Introduction:

Cholesterol is the sole precursor for steroidogenesis, a process that is initiated at the mitochondria with the transfer of cholesterol from the OMM to the inner mitochondrial membrane (IMM) where it is converted to pregnenolone by C27 cholesterol side chain cleavage cytochrome p450 enzyme (P450sc; CYP11A1) (2;3;135). The rate limiting step in this process has been shown not to be the activity of the enzyme Cyp11A1, but substrate availability. As mitochondria are cholesterol poor organelles, cholesterol must be imported into the mitochondria for acute steroidogenesis to continue to occur. This is proposed to be accomplished through specific protein-protein interactions that occur in the cell, specifically between mitochondrial translocator protein (TSPO, 18 kDa), Golgi protein PAP7, the regulatory subunit RIα of cAMP- dependant protein kinase A (PKA-RIα) and the Steroidogenic acute regulatory protein (StAR), which will be discussed below.

The most studied non-vesicular mechanism of cholesterol transport to the mitochondria occurs through the Steroidogenic Acute Regulatory (StAR) protein, a 37 kDa protein whose expression parallels steroid production in adrenals and gonadal cells in exposure to trophic hormones (69-71). Newly synthesized StAR contains an N-terminus mitochondrial targeting sequence which targets the protein to the mitochondria where its activity is proposed to occur. Upon import into the mitochondrial matrix the N-terminus is cleaved, producing the 30 kDa inactive protein. StAR’s C-terminus contains a StAR-related lipid Transfer (START) domain,
an amino acid motif shown to function in cholesterol and lipid binding. As StAR’s mechanism in cholesterol transport is currently not known, it is proposed to act through the C-terminus START domain in which cholesterol is transferred from the protein to the OMM for further import into the mitochondria.

TSPO has been shown to bind cholesterol present in the OMM. Upon treatment of steroidogenic cells with ligands binding specific to TSPO this cholesterol is converted into pregnenolone in both whole cells and isolated mitochondria. It has also been shown that after hormonal stimulation, TSPO’s distribution in the mitochondria is altered to form clusters of 4-6 molecules, which increase ligand binding and translocation of cholesterol to the IMM. Moreover, in TSPO-depleted cells steroidogenesis is greatly reduced and StAR is not processed into the 30 kDa form.

To further identify proteins that interact with TSPO, a yeast-two hybrid screen was performed using TSPO as the bait and several PBP-associated proteins (PAPs) were identified. Of the identified PAP proteins, PAP7 showed the same tissue specificity as TSPO and localized to mitochondria and the Golgi. If was further identified that PAP7 bound PKA-RIα in a yeast-two hybrid screen using PKA-RIα as the bait. From these observations, it was proposed that PAP7 could function by bring PKA-RIα into closer proximity to the mitochondria, where it would interact with proteins shown to play a role in steroidogenesis, specifically
StAR. Formation of a scaffolding complex that could then control the rate which steroidogenesis occurs by regulating the activity of the components of this complex.

In this study we use MA-10 cells which produce maximal progesterone production in two hours following hormonal stimulation. We show through the use of cross-linkable amino acids that this complex, consisting of TSPO, PAP7, PKA-RI, and StAR, forms in both hormonally- and non-hormonally-stimulated cells. We also show that when incubated with cross-linkable cholesterol, it is present in the complex, implying that cholesterol can be delivered to the mitochondria through this complex.
**Material and Methods:**

*Cell Culture:* MA-10 Leydig cells were a gift from Mario Ascoli (University of Iowa, Ames) and were maintained in DMEM/Ham’s F12 (50:50) supplemented with 5% fetal bovine serum (FBS) and 2.5% horse serum at 37 °C and 3.7% CO₂. COS-F2-130 cells were a gift from W.L. Miller and were maintained in Dulbecco’s Modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum.

*cDNA and Plasmid Construction:* cDNA sequences from *Pap7, Tspo, PkaI*, and *Star* genes were obtained as previously stated (138). Briefly, total RNA from MA-10 cells was isolated using TRIzol reagent (Invitrogen), reversed transcribed and amplified with Advantage 2 PCR polymerase (BD Bioscience). The resulting products were sequenced at the Georgetown Sequencing Core Facility and were inserted into the stated expression vectors (BD Bioscience).

*Photo-cholesterol:* A UV spectrum of the sample was taken with 1mL of .5mg/ml solution of photomethionine used, identifying a peak at 345 nm. The half-life of the photomethionine was then determined by exposing the sample to UV-irradiation through a UV light 100-watt mercury lamp (Olypmus) with a peak at 365 (Dapi filter) on IX51 Olympus microscope, a distance of 4 cm from the source to the sample, and measuring the decay of the absorption peak. The UV-irradiation time was determined at four times the half life of the sample, 16 minutes.

*Transient Transfection and Photolabeling:* MA-10 cells were plated in a 6-well plate and 24 hours later were transfected with pEGFP-TSPO and media deficient in
both leucine and methionine. Photoleucine and photomethionine, generated by Dr. Thiele (Max Plank Institute, Dresden, Germany) were added to the media at a concentration of 4mM and incubated for 24 hours. In some cases 5 µCi (83.5pmol) of 7-azi-5α-[3,5,6-3H] cholestan-3β-ol ([3H]photocholesterol), American Radiolabeled Chemicals Inc. (St. Louis, MO) was added to the media along with the photolabeled amino acids, photo-leucine and photo-methionine, and incubated for 24 hours. After 24 hours, cells were treated with 50ng/mL hCG for the stated periods of time, washed with PBS and irradiated with UV light 100-watt mercury lamp (Olypmus) with a peak at 365 nm for 16 minutes.

Cells were then harvested and run on a 4-20% SDS-Page gel, transferred and probed using either antibodies for TSPO, Pap7, StAR, VDAC1, ANT, or GAPDH. COS-F2-130 were also transfected 24 hours after plating in a 6-well plate with StAR cDNA by Lipofectamine 2000 (Invitrogen), photoleucine and photomethionine were both added as stated before. After 24 hours, the cells were washed and irradiated with the UV light, as stated before, harvested and run on a SDS-Page gel, transferred and analyzed as stated previously.
Results:

*Cross-linking amino acids identifies high molecular weight complex:* TSPO, PAP7, PKA-R1α, and StAR have been shown to play a role in steroidogenesis; therefore, to confirm these proteins interact at the OMM, photoactivatable amino acids, specifically leucine and methionine, were used (Fig 5A). These amino acids are functionally incorporated in proteins by the addition to the media, which can then be cross-linked by exposure to UV light. The cross-linked proteins can then be visualized via immunoblotting, allowing identification of protein-protein interactions (139). The immediate cross-linking of proteins permits the identification of transient protein-protein interactions, which normally could not be observed using classical procedures such as immunoprecipitation. To examine the presence of transient protein-protein interactions in steroidogenic cells, twenty four hours after transfection of PAP7 in the presence of photo-amino acids in MA-10 cells were exposed to UV light and immunoprecipitated with anti-GFP. A 100 kDa complex containing both PKA-R1α and TSPO was identified that was only present with the addition of photo-leucine in the presence of cAMP (Fig 5B).

To further confirm complex formation in response to stimulation of steroidogenesis, StAR cDNA was transfected into COS-130-F2 cells, a stably transfected cell line expressing a CYP11A1-adrenodoxin-adrenodoxin reductase fusion protein, in the presence of photo-amino acids. After UV cross linking the
amino acids, immunoblot analysis identified a 210 kDa complex containing immunoreactive TSPO and StAR proteins (Figure 6A).

The presence of StAR suggested that this complex plays a role in steroidogenesis, we next determined if cholesterol was present in the identified complex. MA-10 cells were transfected with GFP-TSPO in the presence of photo-amino acids and after 24 hours, radiolabeled $[^3\text{H}]7$-azi-5α-cholestan-3β-ol ($[^3\text{H}]\text{photo-cholesterol}$) was added to the media for an additional 24 hours. The $[^3\text{H}]\text{photo-cholesterol}$ functions similarly to the photoactivatable amino acids, cross-linking upon exposure to UV light. Following 24 hours of exposure, the MA-10 cells were stimulated with 50 ng/ml hCG to stimulate steroidogenesis and harvested. To determine the amount of photo-cholesterol that had incorporated into the protein complex, the GFP-TSPO was immunoprecipitated and liquid scintillation spectrometry was performed (Figure 6B). The results show a three-fold induction of photo-cholesterol present in the protein complex over basal after hormonal stimulation.

To demonstrate what other protein are present in the identified high molecular weight complex, MA-10 Leydig cells grown for 24 hours in the presence of photo-amino acids were treated with hCG between 10 and 120 min, and cross-linked under UV light. Immunoblot analysis of the proteins identified a 240 kDa protein complex in which TSPO, PAP7, PKA-RIα, and VDAC were observed to be associated in a time-dependent manner following hCG exposure (Fig 6C). StAR
levels peaked after a 30-min treatment with hCG and decreased after two hours of stimulation, while VDAC levels increased slightly and then also decreased at two hours (Fig 6D).
Discussion:

The results presented here demonstrate that a protein complex interacting at the OMM plays a role in the delivery of cholesterol to the mitochondria for optimal steroidogenesis. Protein complex formations are an important and necessary cellular mechanism for the propagation of signals in the cell. A prominent example is the way cAMP achieves signal specificity, cAMP stimulates the activity of protein kinase A (PKA), which phosphorylates downstream signaling substrates. In recent years, we have come to understand that PKA achieves signaling specificity through interactions with A kinase anchoring proteins (AKAPs), which bind to PKA and target it to specific substrates (140). We propose that hormonal signaling in steroidogenic cells achieves cAMP signaling specificity through the formation of a protein complex between TSPO, StAR, Pap7 and PKA-RIα. The formation of this protein complex can ensure the fidelity and amplification of cAMP signal by localizing all necessary proteins that play a role in further enhancing the cAMP signaling and assisting with the transfer cholesterol to the mitochondria. We propose that TSPO functions by anchoring PAP7. The anchoring of PAP7 to the OMM provides a scaffolding mechanism for PKA-RIα to bind. This activity brings PKA-RIα close to the OMM and allows it to interact with StAR, phosphorylating it and increasing its activity. As TSPO, PAP7 and PKA-RIα protein concentrations stay relatively constant during the two hour time frame steroidogenesis is monitored, the data demonstrate that these proteins can make up a basal complex located on the
OMM. As the levels of StAR protein change during the examined period (Fig 6C, D), we propose that the hormonally-regulated StAR activates system. PKA-RIα plays a role in the phosphorylation of StAR, which has been shown to increase its activity at the OMM, demonstrating that the formation and function of the protein complex at basal state can also regulate the activity of complex (141). StAR has been shown to be dependent upon VDAC for import (142), therefore it is interesting to note that VDAC also undergoes depletion during the hCG response parallel to StAR (Fig 6C, D). This finding suggests that VDAC could play a role in the acute response to hormonal stimulation, assisting in the binding of StAR to the OMM. The complex formation would then allow StAR to be phosphorylated, stimulating steroidogenesis. These experiments demonstrated that the inducible hormone complex of steroidogenic proteins, interact to facilitate the transfer of cholesterol from the OMM to the IMM. This complex occurs at the OMM, not at the IMM, as confirmed by presence of VDAC and the absence of ANT in the cross-linked complex.
CHAPTER 2:

TARGETING AND INSERTION OF THE CHOLESTEROL-BINDING TRANSLOCATOR PROTEIN INTO THE OUTER MITOCHONDRIAL MEMBRANE

The work herein has been published in: Malena Rone, Jun Liu, Josip Blonder, Xiaoying Ye, Timothy D. Veenstra, Jason C. Young and Vassilios Papadopoulos. Targeting and Insertion of the Cholesterol-Binding Translocator Protein into the Outer Mitochondrial Membrane. *Biochemistry* 2009; 48(29): 6909-20
Abstract:

Translocator protein (18 kDa, TSPO) is an outer mitochondrial membrane (OMM) protein necessary for cholesterol import and steroid production. We reconstituted the mitochondrial targeting and insertion of TSPO into the OMM to analyze the signals and mechanisms required for this process. Initial studies indicated a formation of a mitochondrial 66 kDa complex through Blue Native-PAGE analysis. The formation of this complex was found to be dependent on the presence of ATP and the cytosolic chaperone Hsp90. Through mutational analysis we identified two areas necessary for TSPO targeting, import, and function: amino acids 103-108 (Schellman motif), which provide the necessary structural orientation for import, and the cholesterol-binding C-terminus required for insertion. Although the Translocase of the Outer Mitochondria Membrane (TOM) complex proteins Tom22 and Tom40 were present in the OMM, the TOM complex did not interact with TSPO. In search of proteins involved in TSPO import, complexes known to interact with TSPO were analyzed by mass spectrometry. The 66 kDa complex formation was found to be dependent on an identified protein, Metaxin 1, for formation and TSPO import. TSPO import into steroidogenic cell mitochondria was increased following treatment of the cells with cAMP. These findings suggest that the initial targeting of TSPO to mitochondria is dependent upon the presence of cytosolic chaperones interacting with the import receptor Tom70. The C-terminus plays an important role in targeting TSPO to mitochondria whereas its import into the OMM is dependent upon
the presence of the Schellman motif. Final integration of TSPO into the OMM occurs via its interaction with Metaxin 1. TSPO import into steroidogenic cell mitochondria is regulated by cAMP.
**Introduction:**

Translocator protein (18 kDa, TSPO), previously known as the peripheral-type benzodiazepine receptor, is an 18 kDa, high affinity, cholesterol- and drug-binding protein that is located in the outer mitochondria membrane (OMM). TSPO appears to contain five α-helixes that span the OMM and assist with the transport of cholesterol from intracellular stores into the mitochondrial matrix, the rate-limiting step in steroid biosynthesis (5;109;131). TSPO has been shown to interact with the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocase (ANT) comprising the mitochondria permeability transition pore (MPTP), which is located at the contact site between the inner and outer mitochondrial membrane (110;113;114;118;143). This mitochondrial localization at the contact site influences many of the biological functions in which TSPO participates, including cholesterol transport, protein import, cell proliferation, and apoptosis (5;100;124;131;144-148). Due to its key role in these cellular functions, the targeting and insertion of TSPO into the OMM is thought to be tightly regulated.

Like the majority of mitochondrial proteins, TSPO is genetically encoded in the nucleus, translated in the cytosol, and then imported into mitochondria (149-151). Unlike many mitochondrial proteins of the matrix and inner mitochondria membrane, OMM proteins such as TSPO, do not have cleavable presequences for mitochondrial targeting. Instead, these proteins are targeted to the OMM through internal amino acid sequences (152). To date, OMM targeting signals have not been
defined or predicted in multimembrane spanning proteins and the signal or signals within TSPO are unknown.

The Translocase of the Outer Mitochondria Membrane (TOM) complex is a protein complex composed of receptors that recognize mitochondrial proteins for import and an aqueous pore for the translocation of proteins across the membrane (153). The TOM complex includes the key components Tom22 and Tom40, the latter of which forms the translocation pore (154). Additional import complexes further direct the protein to its correct location in the OMM, inner mitochondrial membrane (IMM), intermembrane space, and mitochondrial matrix. The TOM receptor protein, Tom70, has been shown to loosely associate with the TOM complex and is important for the import of IMM metabolite carriers that have internal targeting sequences as well as larger hydrophobic proteins (155). Tom70 functions as a docking protein for both Hsc70 and Hsp90 through a central tetratriacopeptide repeat (TPR) domain (156), while its C-terminus is thought to bind mitochondrial proteins during import. Cytosolic chaperones, in particular Hsc70 and Hsp90, have been shown to assist with mammalian mitochondrial import, maintaining the newly made protein in a soluble, import-competent state (156,157). The chaperones that bind the mitochondrial protein dock onto Tom70; the mitochondrial protein is then transferred in an ATP-dependent manner to the core TOM complex for translocation (158).
The import of OMM proteins is an active area of investigation, as these proteins appear to use a diverse array of import mechanisms. C-terminal, tail-anchored OMM proteins, such as Bax and Bcl-xL, which span the OMM once by a transmembrane α-helix, appear to require neither chaperones nor ATP for insertion (159). β-barrel OMM proteins, such as VDAC, require an additional OMM protein complex in addition to the TOM complex, the sorting and assembly machinery (SAM) complex, for correct insertion(160-162). The proposed mammalian SAM complex proteins include Sam50, the pore forming protein of the complex, Metaxin 1, which assists with protein integration into the OMM, and Metaxin 2, a cytosolic protein shown to bind to Metaxin 1(163-165). The stoichiometry and stability of the SAM complex has not yet been firmly established. As TSPO appears to span the OMM via five α-helixes; it is unknown whether TSPO is inserted directly into the OMM through the TOM complex or requires sorting through the SAM complex.

Previously, the results of Otera, et al. (166) have shown that TSPO does not use the traditional protein insertion pathway, but do not identify the pathway necessary for import. Our results presented herein further these studies by demonstrating that (i) during translocation to the OMM, TSPO interacts with cytosolic chaperones to facilitate an interaction with TOM70, (ii) there are specific amino acids necessary for the targeting of TSPO to the OMM, and (iii) once targeting is complete, TSPO insertion into the OMM is mediated through Metaxin 1, a member of the SAM complex. Interestingly, TSPO import into mitochondria was
increased following treatment of hormone-responsive steroidogenic Leydig cells with cAMP, an event that parallels increased cholesterol transport and steroid formation by the cells (124). These findings support the existence of a novel three-step integration pathway for OMM proteins and suggest that protein import into steroid synthesizing mitochondria might be a cAMP- and thus hormone-regulated process.
Material and Methods

Cell Culture: HeLa cells (Lombardi Comprehensive Cancer Center Cell Culture Facility, Georgetown University), a well-established model used to study protein import into mitochondria, were maintained in DMEM supplemented with 10% FBS at 37 °C and 6% CO₂. MA-10 mouse tumor Leydig cells, a well established model to study cholesterol transport into mitochondria and steroidogenesis, were a gift from Mario Ascoli (University of Iowa, Ames) and were maintained in DMEM/Ham’s F12 (50:50) supplemented with 5% fetal bovine serum (FBS) and 2.5% horse serum at 37 °C and 3.7% CO₂. In some experiments, confluent MA-10 cells were used to evaluate the effect of cAMP on mitochondria import. MA-10 cells were treated for two hours with 1mM 8-Bromo cAMP (Biomol, Farmingdale, NY) prior to mitochondrial isolation.

Plasmid Construction: The mouse TSPO cDNA coding sequence (114) was subcloned into pEGFP (CLONTECH Laboratories, Inc., Otsu, Shiga, Japan) at the Sac₁ and BamHI sites (pEGFP-TSPO). For construction of TSPO with a truncated N-terminus, the QuickChange Site-Directed Mutagenesis Kit (Stratagene; La Jolla, CA) was used to generate a Sac₁ mutation in the open reading frame of TSPO at the specified region. The vector was then digested with Sac₁, gel purified, and ligated. TSPO with a C-terminal truncation was generated by creating a sequence verified stop codon mutation at amino acids 151 and 157. For generating TSPO constructs carrying various deletions, the appropriate regions of TSPO were amplified by PCR,
gel purified, ligated, and reinserted into the pEGFP vector. The fusion sites were verified by sequencing.

Confocal Microscopy: Confocal experiments were performed as stated previously (138). Briefly, MA-10 cells were grown on cover glass bottom dish, (Fluorodish, WPI; Sarasota, FL) until 70% confluence. Plasmid constructs were transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA). After 24 hours, cells were stained with 50nM Mitotracker CMX (Molecular Probes; Carlsbad, CA) for thirty minutes and viewed through an Olympus Fluoview FV1000 Laser Confocal Microscope.

Mitochondria Isolation: Mitochondria were isolated by differential centrifugation as previously described (94). Briefly, HeLa cells or confluent MA-10 cells were washed twice with PBS, harvested in Buffer A (10mM HEPES-KOH, pH 7.5, .2 M Mannitol, .07 M Sucrose, 1mM EDTA, 1x Complete Protease Inhibitor Cocktail Tablet (Roche, Switzerland)) using a cell lifter, and centrifuged at 500 x g for 10 minutes. The cell pellet was resuspended in 5 volumes of Buffer A, incubated at 4°C for 10 minutes and then centrifuged at 500 x g for 10 minutes. The cell pellet was resuspended in 5 volumes Buffer B (40 mM HEPES-KOH, pH 7.5, 500mM sucrose, 160 mM Potassium Acetate and 10mM Magnesium Acetate, 1x Complete Protease Inhibitor Cocktail Tablets) and homogenized using an electric potter (glass-Teflon) for 10 passes. Once complete, cells were centrifuged at 500 x g for 10 min. The cell pellet was resuspended in 5 volumes Buffer B with a glass-glass homogenizer (20 passes) and centrifuged at 500 x g for 10 min. The supernatant was pooled and
centrifuged at 10,000 × g for 10 minutes at 4 °C to form a mitochondrial pellet. The mitochondrial pellet was resuspended in 1mL Buffer B and centrifuged at 10,000 × g for 10 minutes to enrich mitochondrial purity. Once complete, the mitochondria were resuspended in mitochondria import buffer (3% BSA, 250 mM sucrose, 5 mM MgCl₂, 80 mM KCl, 10 mM MOPS-KOH, pH 7.2, 5 mM ADP, and 10mM succinate (Sigma, St. Louis), 1x Complete Protease Inhibitor Cocktail Tablets) to give a final concentration of 1 mg/mL mitochondria for BN-Page import and 5mg/mL mitochondria for sodium carbonate extraction. Mitochondria were kept on ice until use for no longer than 1 hour.

Protein Import: Radiolabeled TSPO was generated using the TNT® T7 Quick Coupled Transcription/Translation System (Promega; Madison, WI) in the presence of [³⁵S]-methionine (Amersham Biosciences; Piscataway, NJ) as performed previously (124) for one hour at 30°C. Once complete, the reaction was terminated by the addition of one volume of 2xTT buffer (20 mM Hepes-KOH pH7.5, 500mM sucrose, 80 mM KOAc, 5mM MgOAc₂, 1mM Methionine). 5 µl of the TNT reaction was added to 50 µg isolated mitochondria in import buffer for the stated times. Mitochondria were centrifuged at 10,000 × g for 10 minutes, solubilized with 1% digitonin buffer (20 mM Tris-Cl, 0.1 mM EDTA, 50 mM NaCl, 10% w/v glycerol, 1% digitonin (Invitrogen) and 1 mM PMSF) for 20 minutes on ice, and centrifuged at 10,000 × g for 10 minutes. One-half of each sample was digested with
250 µg/ml proteinase K (Qiagen; Dusseldorf, Germany) at 4 °C for 10 minutes while the other half of the sample remained untreated.

**Blue Native (BN)-PAGE**: BN-PAGE was performed as described by Simpson (2). BN-PAGE loading dye (5% w/v Coomassie Brilliant Blue G-250, 500 mM ε-amino-n-caproic acid, and 160 mM BisTris, pH 7.0) was added to the sample supernatants, loaded onto a 4-16% native gel (Invitrogen), and run at 130 V. Once complete, the gel was transferred using a semi-dry transfer apparatus for one hour at 25 V. The PVDF membrane was then fixed and dried. Then membrane was exposed to a multipurpose phosphor screen for 1-7 days and analyzed by phosphorimaging using the Cyclone Storage Phosphor System (Packard BioScience; Waltham, MA). Once complete, the membrane was then used for immunodetection. Image analysis was performed by Multi Gauge V3.0 from FujiFilm.

**Carbonate Extraction**: Mitochondrial protein import was analyzed as described by Fujiki et al. (167). After import, mitochondria were incubated at 0.5 mg/ml in 180 µl of 0.1 M Na₂CO₃ (pH 10.5) at 4 °C for 30 minutes. Forty µl of sucrose buffer (500 mM sucrose and 0.1 M Na₂CO₃, pH 10.5) was added to the top of the sample and centrifuged at 180,000 × g for 30 minutes at 4 °C in a Beckman Coulter TLA-100 rotor. Trichloroacetic acid precipitation was performed on the supernatant, and both pellet and supernatant were analyzed by SDS-PAGE.

**Nickel-Sepharose Pull Down Assay**: The phosphate carrier (PiC) was also generated from cell free transcription/translation reactions as stated previously (168).
Radiolabeled TSPO and PiC were diluted 10-fold with reticulocyte lysate and incubated with either geldanamycin or novobiocin. Tom70-H3 was then added to the TNT reaction of either PiC or TSPO (156). After five minutes, apyrase was added to terminate the incubation reaction and 50\(\mu\)l of GTI buffer (100mM KOAc, 20mM Hepes-KOH pH7.5, 5mM MgOAc\(_2\), 20mM imidazole, 0.1% Triton X-100) was added. Nickel-Sepharose slurry was added to the mixture and incubated on an orbital shaker at 4°C for 30 minutes. The resulting reactions contained 5% reticulate lysate reaction of TSPO or PiC containing the nickel-Sepharose slurry, the negative control reaction did not contain Tom70-H3, positive control, geldanamycin, and novobiocin treated samples contained 5\(\mu\)M Tom70-H3 and either 18\(\mu\)M geldanamycin or 1mM novobiocin. The beads were then washed twice with GTI buffer and once with GI buffer (lacking Triton X-100). The protein was eluted with 40\(\mu\)l LLB with EDTA and separated by SDS-PAGE.

**Steroid Biosynthesis:** Steroid analysis was performed as previously described (138). Briefly, MA-10 cells were plated in 24-well plates at a density of 50,000 cells/well. Cells were transfected after 24 hours with various GFP-TSPO constructs. Culture media were collected 24-48 hours post-transfection. In certain experiments, cells were washed with serum-free media and treated with 50 ng/ml hCG for two hours to determine the hormone-responsiveness of the cells. At the end of the incubation, media were collected. To determine maximal steroid production, the hydrosoluble substrate 22R-hydroxycholesterol (10 \(\mu\)M) was added for two hours to the cells.
cultured in serum-free media. Progesterone production was measured by RIA in the media. Anti-progesterone antiserum was from MP-Biomedicals (Solon, OH) and [1,2,6,7-\textsuperscript{3}H(N)]-progesterone (specific activity: 17.5 Ci/mmol) was from PerkinElmer Life Sciences.

In-gel Digestion and MS Analysis: Selected protein spots from HeLa extracts separated by BN-Page followed by 2D-SDS-PAGE were subjected to an in-gel digestion procedure as described elsewhere (169). Resulting in-gel digests were desalted using C18 Zip Tips (Millipore) before analysis by nano-flow reversed-phase liquid chromatography (nanoRPLC) using an Agilent 1100 LC system (Agilent Technologies, Inc., Paolo Alto, CA) coupled online to a linear ion trap (LIT) mass spectrometer (LTQ, Thermo Scientific, San Jose, CA). Reversed-phase separations were performed using 75 µm i.d. × 360 µm o.d. × 10 cm long capillary columns (Polymicro Technologies Inc., Phoenix, AZ) that were slurry packed in-house with a 5-µm, 300 Å pore size Jupiter C-18 silica bonded stationary phase (Phenomenex Torrance, CA). After being injected with 5 µl of sample, the column was washed for 20 minutes with 98% solvent A (0.1% formic acid in water, vol/vol) and peptides were eluted using a linear gradient of 2% solvent B (0.1% formic acid in 100% acetonitrile, vol/vol) to 85% solvent B for 110 minutes at a constant flow rate of 250 nl/min. The LIT-MS was operated in a data-dependent mode in which each full MS scan was followed by seven MS/MS scans where the most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID).
using a normalized collision energy of 36%. The temperature of the heated capillary and electrospray voltage (applied on column base) was 180 °C and 1.7 kV, respectively. The CID spectra were searched against a non-redundant human protein database using SEQUEST (Thermo Scientific, San Jose, CA) and results were tabulated for each identified peptide/protein.

Transfection with siRNA: Metaxin 1 siRNAs

(5’-GCGCUGUCCUCAGAAUAAACCUGTT-3’;
5’-CGUAAAGAGAAGUAUAAUGCCGACT-3’;
5’- GGAUAGACGCCAAGAACUAUGUGGA-3’) were purchased from IDT (Coralville, IA) in a TriFecta kit. A hypoxanthine-guanine phosphoribosyltransferase (HPRT)-targeted positive control and a scrambled negative control were obtained from the same provider. MA-10 cells cultured in 100-mm dishes were transfected with 30 µl Lipofectamine RNAiMAX (Invitrogen) and 20 nM total of either the control siRNA duplex or protein-targeted duplexes for 48 hours. At the end of the treatment, media was changed and 3 days later cells were harvested and mitochondria were isolated for import studies.

Real-Time qPCR: 1x10^5 cells were harvested from the control and 8-Bromo cAMP treated MA-10 cells and used for RNA isolation to measure stated mRNA levels. RNA was isolated with an RNeasy Mini Kit (Qiagen) with optional DNase Digestion. The cDNA was then generated from Advantage RT-for-PCR kit (Clontech), using 100ng RNA incubated with oligo(dT) primer. Analysis was
performed with 7900HT Sequence Detection Agents (Applied Biosystems), primers used are stated in Table 1, TaqMan™ was from Applied Biosystems (Foster City, CA). All sequences were normalized to HPRT.

Statistics: Statistical analysis was performed using Prism version 4.0 (GraphPad Software, San Diego, CA). Group means were compared using student's t test or two-way ANOVA test followed by a Bonferroni column test. Data are presented as mean ± sem, $p < 0.05$ was considered significant.

Materials: Antibodies specific for TOM22 (Sigma), VDAC, Cox IV, Tom40 (Abcam; Cambridge, MA), Metaxin 1 (BD Bioscience; San Jose, CA) GapDH (Trevigen, Gaithersburg, MD) were purchased from the various vendors. StAR antibody was a generous gift from Dr. DB Hales (170). A specific rabbit polyclonal antibody was raised against the purified cytosolic fragment of human Tom70. The TSPO polyclonal antibody was developed as previously described (96).
Results

Import of TSPO – To identify the import pathway of TSPO, we incubated cell-free, radiolabeled, in vitro translated TSPO with isolated HeLa cell mitochondria and monitored the formation of radiolabeled protein complexes through BN-PAGE by phosphoimaging. We have previously shown that the incubation of TSPO with isolated mitochondria results in import of a fully functional protein, capable of binding and transporting cholesterol in isolated steroidogenic mitochondria from MA-10 cells with consequent generation of steroids (124). As the TSPO import reaction with isolated mitochondria proceeded, we observed the presence of a radioactive band migrating at approximately 66-kDa that increased in intensity with incubation time (Fig. 7A). A denaturing 2D SDS-PAGE of the BN-PAGE import reactions produced an 18 kDa radioactive band, further confirming TSPO import into the mitochondria (data not shown). The presence of the TOM complex in the mitochondria was assessed by immunoblot analysis of proteins separated on BN-PAGE gels, electrotransferred on membranes, and blotted using antibodies against Tom22 and Tom40. Both of these antibodies recognized immunoreactive proteins that migrated at 440 kDa, thus indicating the presence of the core TOM complex at the appropriate size though TSPO was not present in the complex. These data were confirmed in experiments in which the major import receptor Tom20 was knocked down; its absence failed to affect TSPO import into mitochondria (data not shown). We further probed native membranes with an affinity-purified, anti-TSPO peptide-
specific polyclonal antibody. Immunoreactive proteins were identified to migrate at 300 kDa and 600 kDa. It should be noted that HeLa cells are known to have low levels of endogenous TSPO. In parallel studies using the rich-in-TSPO MA-10 cell mitochondria we identified immunoreactive TSPO at 66 kDa, 300 kDa and 600 kDa (data not shown). As mature TSPO is known to form polymers (107) and also associate with various other IMM and OMM proteins (113;114), these large complexes likely contain both homo- and/or hetero-polymers of TSPO. The 66 kDa protein complex formed by newly imported TSPO was not detected in HeLa cells by immunoblot analysis as seen of steady-state endogenous TSPO. This may be due to the low levels of TSPO generated by the TNT radiolabeling kit, it must be remembered that TSPO antibodies directed towards the mature peptide are unlikely to recognize protein morphology altered during mitochondrial import.

To confirm TSPO import into isolated mitochondria, we performed a proteinase K digestion to degrade non-imported proteins. Protein import was performed at 4°C and 33°C and import reactions were terminated at 0 and 30 minutes. Samples were then divided into two sets, with and without proteinase K treatment. With the 4°C import reactions, the amount of 66 kDa complex was greatly reduced relative to reactions at 33°C. Proteinase K digestion of the 4°C reactions removed the 66 kDa protein complexes that were observable in untreated samples (Fig. 7B). In contrast, with the permissive 33 °C-import reaction, the 66 kDa complex was resistant to
proteinase treatment (Fig. 7B). These data suggest that the 66 kDa complex, under permissive conditions, is fully incorporated into mitochondrial membranes.

Further confirmation of TSPO membrane integration was obtained by carbonate extraction. Here TSPO was imported under permissive conditions and mitochondria were purified. These re-isolated mitochondria were treated with sodium carbonate to remove proteins that were associated with, but not integrated into, the OMM. Upon carbonate treatment and centrifugation, the majority of TSPO was found in the pellet with the mitochondrial membrane fraction, implying full incorporation of the protein into the OMM (Fig. 7C). As a positive control, the carbonate extraction experiment was performed with radiolabeled Tom70, an integral OMM protein, which displayed the same association profile as TSPO (Fig. 7C). In contrast, the soluble matrix protein Hsp60 was mostly found in the extracted supernatant fraction. Thus, the HeLa mitochondria have the necessary machinery to drive authentic TSPO import.

**TSPO associates with HSP90 and Tom70** – Heat shock proteins (HSPs), including Hsp90, are known to play an important role in delivering proteins to the OMM for import, interacting with Tom70 and assisting translocation in an ATP dependent manner (158). Therefore, to determine if TSPO interacts with Hsp90 for import into the OMM we incubated the purified C-terminal fragment of Hsp90 (C90) with TSPO import reactions into isolated mitochondria. C90 has previously been shown not to interact with mitochondrial proteins before import but does bind stably to Tom70, which outcompetes the HSP90 interaction and inhibits Tom70-dependent import.
The addition of C90, resulted in a large reduction in TSPO import compared to control (Fig. 8A, C), suggesting that import is dependent upon Hsp90 interaction. To further test this hypothesis, we performed a series of BN-PAGE experiments. Considering that the chaperone-Tom70 pathway requires ATP for the function of Hsp90 or Hsc70, we depleted ATP from import reactions using apyrase. BN-PAGE analysis of the samples indicated that the membrane-integrated 66 kDa complex was reduced dramatically in the early stages of import (Fig. 8B). As Hsp90 seemed to function in TSPO targeting to Tom70, we next used the specific Hsp90 inhibitors geldanamycin (GD) and novobiocin (NB) to directly confirm the role of Hsp90 in TSPO import. GD obstructs the N-terminal ATP-binding domain of Hsp90 and inhibits the ATPase activity of the protein, resulting in a stalled complex and decreased translocation across the OMM (158;171). NB interferes with the targeting of proteins to the mitochondria by binding near the C-terminus of Hsp90, inhibiting substrate binding (172). NB also interferes with the docking of Hsp90 to Tom70. As shown in Figures 8B & C, NB and GD significantly reduced import of TSPO compared to control (p<0.01 by ANOVA). NB shows the greatest decrease in import; this is expected as NB would prevent TSPO from binding to Tom70 and associating with the mitochondria. GD is decreased, though not to the same extent as NB, caused by the prevention of cleavage of ATP, thus stalling TSPO on the mitochondrial membrane.
To further confirm that TSPO interacts with Tom70 for import, radiolabeled TSPO was incubated with the His-tagged Tom70 cytosolic fragment and then co-precipitated with nickel-sepharose, reconstituting the targeting step as previously published. TSPO was recovered from the nickel-sepharose only when His-tagged Tom70 was present, indicating that TSPO interacts with Tom70 for import (Fig. 8D). GD and NB were incubated separately with the His-tagged Tom70 fragment and radiolabeled TSPO, and Tom70-associated material analyzed. GD did not inhibit the binding of TSPO to Tom70 as the recovery levels were similar to the control (Fig. 8D). This finding was similar to that observed with Hsp90-dependent precursor proteins, as GD only inhibits the ATP-dependant release of TSPO from the bound chaperone-Tom70 complex. In contrast, NB did prevent TSPO from associating with Tom70. Again, this finding matched the behavior of Hsp90-dependent precursors. Our results suggest that Hsp90 functions to target TSPO to the Tom70 import receptor by a mechanism typical of the chaperone-Tom70 pathway.

*N-terminus is necessary but not sufficient to target TSPO to the mitochondria* - To identify the OMM-targeting sequence(s) of TSPO, we tested the localization of GFP-fused TSPO in MA-10 mouse Leydig cells. TSPO was tagged on either the N- or C-terminus to confirm that the presence of GFP did not interfere with the localization of the protein to mitochondria (data not shown). We then chose a construct in which GFP was linked at the N-terminus of TSPO to ensure that the cholesterol-binding domain on the C-terminus of the protein (96;173) would not be directly affected by
the presence of the GFP moiety. A series of deletion constructs were generated to determine whether the N-terminus is important for mitochondrial targeting (Fig. 9). Constructs in which the first 7, 28, 48, 68, and 85 amino acids were removed showed only a slight decrease in TSPO co-localization with the mitochondria, labeled with the Mitotracker CMX dye (Fig. 9A-F). Removal of amino acids 1-110 resulted in a loss of co-localization of TSPO with mitochondria and appearance of a diffuse pattern, consistent with an import defect (Fig. 9G).

As this region appeared necessary for TSPO import, we next examined the importance of amino acids found between residues 88-110. A Schellman motif was predicted between amino acids 103-108. This motif, often found terminating α-helical secondary structures, is stabilized through hydrophobic interactions between amino acids around a signature glycine residue (174). In TSPO, the Schellman motif is predicted to reside in the second intermembrane space loop, bridging the third & fourth predicted transmembrane helices (Fig. 9a). Removal of these amino acids (Δ103-108) resulted in focal aggregates of GFP-TSPO in areas adjacent to the mitochondria, as seen through a decrease in co-localization with Mitotracker (Fig. 9H). The point mutation of glycine 106 to alanine (G106A), that disrupts the Schellman motif by reducing flexibility, resulted in a similar profile as seen with TSPOΔ103-108 (Fig. 9I). The Schellman motif between amino acids 103-108 of TSPO thus appears to be critical for proper folding and/or membrane insertion. Though its role in localization and import remain open to investigation, the
juxtamitochondrial location of aggregates suggests that the proteins are targeted, but not capable of import.

_C-terminus is necessary for targeting to the OMM_ - As roughly two-thirds of the TSPO protein from the N-terminus can be removed without affecting the localization of the remaining protein to mitochondria, we then questioned whether the removal of the C-terminus affects TSPO localization. Generation of Δ151-169 and Δ157-169 amino acid-deletion constructs caused a decrease in the localization of the protein to mitochondria with an increase in aggregation of GFP-TSPO (Fig. 9J, K).

Considering that the cholesterol-binding domain of TSPO is present within the deleted region in TSPO Δ151-169, it was possible that this domain could play a role in the targeting of TSPO to the OMM. However, removal of region 148-157, which contains the cholesterol-binding domain, did not alter the TSPO/mitochondria co-localization pattern (Fig. 9L), suggesting that the domain does not play a role in TSPO import. Because the degree of TSPO/mitochondria co-localization decreases with the removal of the C-terminus and although the cholesterol binding domain plays no role, it could be that some other feature of the C-terminus, such as the overall secondary structure, could play a role in TSPO localization with the OMM. A comparison of the Schellman motif and C-terminal sequences was made, revealing two conserved areas of high leucine content, amino acids 112-114 and 136, 137, and 141 (Fig. 10A). Point mutations of both sets of leucines (Leucine$_{112-114}$ and
Leucine$^{136,137,141}$ into alanines did not alter TSPO co-localization with mitochondria (Fig. 9M, N).

Inhibition of import of TSPO mutants - In the above-described GFP-TSPO/mitochondria co-localization experiments, several constructs displayed reduced degrees of co-localization. To confirm that reduced co-localization reflects reduced TSPO import into mitochondria, BN-PAGE of in vitro import reactions was performed using [$^{35}$S]-TSPO mutant constructs. Notably, the Schellman motif mutant Δ103-108 could not be imported to the 66 kDa complex (Fig. 10B). The import of TSPO carrying the G106A point mutation also resulted in the greatly decreased import of the 66 kDa protein complex, although not to the extent seen with the Δ103-108 TSPO construct (Fig. 10B). The ability to import was then tested for the TSPO constructs in which the C-terminal domains, 151-169 and 157-169, were removed. Both deletions clearly abolished the import of the 66 kDa complex in isolated mitochondrial (Fig. 10C). To determine whether the alteration of the structure of the C-terminus results in the inhibition of TSPO import, the Leucine$^{112-114}$ and Leucine$^{136,137,141}$-TSPO point mutants were analyzed by BN-PAGE. Leucine$^{112-114}$ did not show an effect on TSPO import (Fig. 10D). However, the Leucine$^{136,137,141}$-TSPO mutant showed a significant decrease in the import of the 66 kDa complex (Fig. 10D) although its level of synthesis in vitro was similar to the Leucine$^{112-114}$ mutant (Fig. 10E). This result differs from the results of confocal microscopy in which the Leucine$^{112-114}$ TSPO mutant was found to co-localize with
mitochondria. This apparent discrepancy could be due to differences between the in situ and in vitro situations, including the highly concentrated intracellular environment, which favor TSPO import and alleviate partial import defects. Overall, the in vitro and live cell results support an important role of the Schellman motif and sequences within the C-terminus of TSPO for import.

Functional evaluation of TSPO import in steroid biosynthesis - As TSPO controls the rate-limiting step in the production of steroids, cholesterol transport into mitochondria, increasing the TSPO protein concentration in the OMM by transient transfection with a TSPO construct was shown to increase progesterone production by MA-10 mouse Leydig cells in culture (138). To investigate the functional role of altered import of TSPO mutants previously evaluated by confocal microscopy and BN-PAGE analyses, we measured progesterone production in MA-10 cells transfected with wild-type and mutated TSPO constructs (Fig. 9). As the constitutive expression of TSPO in MA-10 cells was not silenced, a basal level of progesterone production was observed. However, transfection of MA-10 cells with GFP-TSPO resulted in a marked 2.5-fold increase in progesterone production (Fig. 10F; \( p < 0.01 \) by Student’s t-test), consistent with previous findings (138). Transfection with TSPO in which the Schellman motif was mutated (G106A) did not affect basal progesterone formation but did not cause a similar increase seen by transfection with wild-type GFP-TSPO. The results obtained with the G106A TSPO mutant support the data obtained in situ by confocal microscopy and in vitro using BN-PAGE.
indicating that such mutants are not imported and therefore not functional in cholesterol transport. The Leucine^{136,137,141} mutant showed progesterone levels near basal levels, suggesting that while this mutant may be imported into the OMM, it was not functional in the transport of cholesterol into the mitochondrial matrix. C-terminal mutants also failed to increase progesterone production, consistent with an import defect. The finding that removal of the cholesterol-binding domain in the TSPOΔ151-169 construct resulted in the reduction of basal progesterone production is in agreement with previous findings (124;173).

Metaxin 1 is necessary for TSPO import - Considering the data presented above that TSPO does not stably associate with the core TOM complex during import, we undertook a detailed analysis of the identification of proteins involved. Using a combination of BN-PAGE and 2D-SDS-PAGE followed by mass spectrometric analysis of proteins present in all spots separated from the 66 kDa complex, as well as the stable 800 kDa complex, we identified a number of proteins of interest (Table 2). Among the identified proteins present in the 66 kDa protein complex was Metaxin 1. Metaxin 1 was shown previously to be present in MA-10 cells and be induced by hormone-treatment in a manner parallel to steroid synthesis (175). Probing BN-PAGE immunoblots with an anti-Metaxin 1 antibody showed that the protein identified migrated at a similar molecular size to $[^{35}\text{S}]$-TSPO (Fig. 11A). Metaxin 1 is the mammalian homolog of yeast Sam37 (also called Mas37/Tom37), a component of the SAM complex known to function in the stable integration of
OMM β-barrel proteins (162;165). Metaxin 1’s primary role in the OMM is to function as a receptor in the SAM complex (176). It was possible that Metaxin 1 could play a role in TSPO import and to address this, an siRNA duplex that targets Metaxin 1 was used to knock down its expression. Mitochondria were isolated from MA-10 cells that had been treated for 5 days with 20 nM of either a siRNA complex toward Metaxin 1 or a scrambled negative control. Figure 11C shows that the siRNA successfully reduced Metaxin 1 protein levels compared to the scrambled siRNA control. Moreover, the basal levels of the inner mitochondrial membrane protein COXIV were unaffected by Metaxin 1 silencing, though endogenous TSPO levels were slightly decreased. BN-PAGE of import into the mitochondria from Metaxin1 knockdown cells showed that the rate of TSPO import at the 66 kDa complex was diminished, compared to mitochondria from scrambled siRNA and untreated cells (Fig. 11B). This data, quantified in Figure 11D ($p<0.05$ by ANOVA), suggests that Metaxin 1 is involved in TSPO import into the OMM.

*Cyclic-AMP stimulated mitochondria increases TSPO import:* Steroidogenesis is initiated upon the transfer of cholesterol from the OMM to the IMM where it interacts with CYP11A1 to be converted into pregnenolone. This transfer of cholesterol is activated via a cAMP-dependent pathway, resulting in an increase in protein synthesis, protein phosphorylation and lipid synthesis (177). To determine if activation of steroidogenesis would alter mitochondrial protein import, MA-10 cells were incubated with 1mM cAMP for two hours, the point in which maximal rates of
steroid production are seen in vitro, and the mitochondria were isolated (138). Upon initiation of TSPO mitochondrial import, a 1.7-fold increase of 66 kDa TSPO was observed in mitochondria isolated from cAMP-treated cells within the first 5 minutes followed by a 2.2- and 1.8-fold induction over basal at 15 and 30 min, respectively (Fig. 12A, B). The effect of cAMP was statistically significant (p<0.05 by t-test). To determine if gene and protein expression levels were altered both qPCR and western blot analysis were performed. qPCR results showed no increase upon cAMP treatment of the Tom40 pore for OMM protein import and Tim22 for IMM protein import. Metaxin 1 showed a slight but not significant increase upon cAMP stimulation (Fig. 12C). While the 2 hour cAMP treatment is too short for most transcriptionally-based changes in protein expression, translation- or degradation-based regulation can cause faster effects. However, upon immunoblot analysis there were no significant increases seen in any mitochondrial proteins including Metaxin1 (Fig. 12D). StAR protein expression, which is a cAMP-induced protein, was also measured to confirm the 8-bromo cAMP activity. StAR mRNA levels were increase by 10-fold (data not shown) and there was also a pronounced induction of StAR protein levels (Fig. 12D). This suggests that while the mechanisms necessary for mitochondrial import are being up-regulated, the increase in TSPO import is not due to an increase in mitochondria import proteins. It is possible however that changes in the composition of protein complexes and protein-protein interactions needed for import could occur.
Discussion

In the above experiments, we determined the pathway for the targeting and insertion of the multi-membrane spanning protein, TSPO, into the OMM. A schematic representation of the steps of the process is shown in Figure 13. The initial targeting of TSPO to mitochondria has been shown to be dependent on the presence of the cytosolic chaperones Hsp90 and most likely Hsc70 as well as their interactions with Tom70 upon arrival at the OMM. As this first step shows, these early interactions of the cytosolic chaperones and Tom70 depend on the proper binding of TSPO to the chaperones. Targeting of chaperone-TSPO complexes to Tom70 is the second step. The Tom70 complex formed at this stage may be relatively unstable and although not detected in the BN-PAGE, it can be reconstituted with purified Tom70. The third step of TSPO import into the OMM is insertion into the membrane. This is a temperature and ATP-dependent process involving the release of TSPO from the chaperones and Tom70. Metaxin1 functions at this step to boost the kinetics of insertion. After insertion, TSPO becomes resistant to proteinase K. The third step requires formation of specific secondary structures including the Schellman motif, which probably aids integration of the third and fourth transmembrane domains of TSPO into the OMM. In steroidogenic and hormone-responsive MA-10 Leydig cells, the rate of import into the isolated mitochondria can be accelerated following treatment with cAMP (Fig. 13; yellow arrow). These conformational requirements suggest that the 66 kDa complex contains TSPO in a native folded state. The native
complex is most likely a homo-oligomer of TSPO as has previously been observed. The 66 kDa complex then matures into the final complexes of up to 800 kDa which are observed in steady-state mitochondria of endogenous TSPO. These complexes may include the known TSPO interacting proteins VDAC and ANT, further identified by mass spectrometry to be present in this complex, as well as other proteins. This process of TSPO import constitutes a novel pathway for protein import into the OMM.

As mentioned earlier, our results broadly support the conclusion of Otera et al. (166) that TSPO is inserted into mitochondria via a novel pathway. The authors identified a 66 kDa TSPO complex by BN-PAGE upon import into HeLa mitochondria. They also demonstrated that Tom22, Tom20, Tom40, and Sam50 were not involved in TSPO import whereas Tom70 played a role in TSPO targeting to mitochondria, in agreement with our findings. They hypothesized the importance of other unidentified mitochondrial proteins for TSPO and other protein import. We now confirm these findings and extend them by providing evidence that Hsp90 and Metaxin1 also function in the targeting and insertion of TSPO into the OMM.

Although the role of heat shock proteins in TSPO mitochondrial import was previously discussed, the interaction was described as ATP-independent, a conclusion that is not supported by our data. Our demonstration of ATP-dependent import is in fact consistent with established work on the Tom70 pathway and with current models of Hsp90 and Hsc70 activity.
We furthermore report that Metaxin1 and the C-terminus of TSPO are critical for TSPO insertion into the OMM. These findings might explain the mechanism of import of a number of proteins into the OMM. Sam37 has been shown to play a role in the import of α-helical, C-terminal-targeted TOM proteins, such as Tom22 and small Tom proteins. This finding suggests that the interaction of TSPO via its C-terminus with Metaxin 1 is necessary for its complete import into the OMM. Slight alteration of the α-helix of the C-terminus could interfere with this import, as seen with the inhibition of Leucine\textsuperscript{136,137,141} mutant import by BN-PAGE. Metaxin 2, a cytosolic protein shown to interact with Metaxin 1 at the OMM, could also play a role in import. Moreover, we identified the amino acids critical for insertion of the protein into the OMM and demonstrated the functional significance of TSPO insertion and import into mitochondria of steroidogenic cells in which TSPO, a cholesterol-binding protein, functions in cholesterol import, the rate-determining step in steroid biosynthesis.

The final step of TSPO import is the functional integration into the OMM. Antibody detection of TSPO on the BN gel membrane identified a major 800 kDa complex while not recognizing the protein migrating at 66 kDa either due to limited amounts of the protein or accessibility of the antibody to the antigen. We propose that the 800 kDa complex represents the biologically functional population of TSPO in the OMM. Mass spectrometric analysis of these complexes revealed that at the 66 kDa complex in HeLa cells, in addition to Metaxin 1, VDAC1 was identified. As
Metaxin1 assists with β-barrel protein insertion into the OMM, it is possible the close association of TSPO with VDAC could form here, allowing a foundation for a larger protein complex to form. Mass spectrometric analysis of the 800 kDa complex identified the isoforms of VDAC and anion nucleotide transporter (ANT). As it is known that TSPO physically and functionally interacts with VDAC (113;114;138) and ANT and that they are part of the mitochondrial permeability transition pore (MPTP) (148), we propose that these complexes represent the contact sites between the OMM and the IMM, a place where TSPO is concentrated (110). As contact sites have been suggested to play a role in cholesterol transport, apoptosis, energy metabolism and protein transport; further understanding of the proteins involved in this area could lead to a deeper understanding of the function of contact sites (178-180). Interestingly, at the 800 kDa protein complex, mass spectrometry identified other known mitochondrial proteins, that is, fatty acid synthase and apolipoprotein AI and AII, likely participating in cholesterol transport into mitochondria, needed for steroid formation in steroidogenic cells and membrane biogenesis in proliferating cells.

In conclusion, the findings reported herein using as a model TSPO support the existence of a novel three-step integration pathway for OMM proteins and suggest that such protein import might be a cAMP-regulated process.
CHAPTER 3:
HORMONAL INDUCTION OF MITOCHONDRIAL PROTEIN COMPLEXES AND STEROID FORMATION IN MA-10 LEYDIG CELLS:
IDENTIFICATION OF A MITOCHONDRIAL PROTEIN COMPLEX CONTAINING HORMONE-INDUCED CHOLESTEROL SIDE CHAIN CLEAVAGE ACTIVITY
Abstract:

Cholesterol transfer from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) is the rate-limiting and hormone-sensitive step in the acute regulation of steroid hormone synthesis. The hormone-induced transfer of cholesterol to the OMM is accomplished by the formation of a multimeric protein complex anchored at the OMM by the translocator protein (18 kDa; TSPO) and composed of the cytosolic proteins; steroidogenic acute regulatory protein, TSPO-associated protein PAP7 and PAP7-associated regulatory subunits of the cAMP-dependent protein kinase, as well as the TSPO-associated voltage-dependent anion channel located at the OMM/IMM contact sites. To evaluate how upon hormonal stimulation cholesterol reaches the IMM enzyme CYP11A1, which cleaves the side chain of cholesterol and initiates steroid synthesis, we analyzed the presence of such complexes in control and hormone-treated MA-10 Leydig cells. We used blue native polyacrylamide gel electrophoresis followed by immunoblot analysis and mass spectrometry to identify protein complexes in control cells. TSPO was found in 66 and 800 kDa protein complexes. Many proteins that belong to both the OMM and IMM were found in the 800 kDa complex suggesting that it may be part of the OMM/IMM contact sites. A 140 kDa complex containing CYP11A1 was identified as well. Upon hormone treatment, however, CYP11A1 was found in the 800 kDa complex. To determine whether the CYP11A1-containing complexes are functional we devised a method using a chemically synthesized CYP11A1 probe,
cholesterol resorufin in which cleavage of cholesterol to form pregnenolone by CYP11A1 releases the fluorescent resorufin. The results obtained demonstrated that under basal conditions the 140 kDa complex contains a functional CYP11A1. Upon hormonal stimulation, this activity moves to the 800 kDa complex and steroidogenesis begins. Taken together these results suggest that acute hormone-induced steroidogenesis begins at the 800 kDa mitochondrial OMM/IMM contact site complex.
Introduction:

The transfer of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) is the hormone-sensitive and rate-limiting step in the production of steroids. This transfer of cholesterol allows for its conversion to pregnenolone by C27 cholesterol side chain cleavage P450 enzyme (CYP11A1). The activity of CYP11A1 was presumed to be the rate-limiting step in steroidogenesis until identification that membrane permeable, hydroxylated analogs of cholesterol, such as 22R-hydroxycholesterol, could produce steroids in the absence of hormone stimulation (181;182). These findings demonstrated that CYP11A1 was fully active in control cells and the availability of the substrate cholesterol and its transfer rate to the IMM, where CYP11A1 and its auxiliary proteins adrenodoxin and adrenodoxin reductase are located was the rate limiting reaction. Therefore, for hormones to rapidly produce the large volume of steroids needed to supply the body a mechanism must occur that facilitates the transfer of cholesterol from OMM to IMM.

As discussed in chapter 1, the hormone-induced transfer of cholesterol to the OMM is accomplished by the formation of a multimeric protein complex anchored at the OMM by the translocator protein (18 kDa; TSPO) and composed of the cytosolic steroidogenic acute regulatory protein (STAR), TSPO-associated protein PAP7 and PAP7-associated regulatory subunits of the cAMP-dependent protein kinase (PKA-
RIα and β, as well as the TSPO-associated voltage-dependent anion channel (VDAC) located at the OMM/IMM contact sites (138).

StAR was initially identified to play a role in steroidogenesis as its synthesis parallels hormone-induced steroid formation (183;184). StAR’s N-terminus contains a mitochondrial targeting sequence while its C-terminus contains a StAR-Related Lipid Transfer (START) domain that binds cholesterol at 30nM affinity, suggesting a mechanism in which it could transfer cholesterol to the mitochondria (76;127). StAR is imported into the mitochondria, due to which it was proposed that it could function in the transferring of cholesterol to the IMM (128). Though, further analysis identified that StAR is primarily active at the OMM and import of the mature protein into mitochondria ends its activity (124). These results suggest that although StAR plays an important role in transferring cholesterol to the OMM, it may not be involved in the transfer of cholesterol to the IMM.

TSPO is a high affinity cholesterol binding protein, which spans the OMM in five α-helixes (83;185). TSPO was identified to assist with the transfer of cholesterol into the OMM and from OMM to IMM (94). It was identified that upon exposure to TSPO drug ligands in steroidogenic cells and isolated mitochondria production of steroids is increased (83;85;186). Targeted deletion of TSPO in Leydig cells stops cholesterol transport into the mitochondria and to the IMM (102). Upon cellular exposure to hCG, TSPO has been shown to polymerize, experiments have shown that dimerized TSPO has lower affinity for cholesterol and higher affinity for TSPO
ligands, suggesting a mechanism for cholesterol translocation into the IMM (107) (131).

TSPO has been previously shown to interact with mitochondrial proteins Voltage Dependant Anion Channel (VDAC) and Adenine Nucleotide Translocase (ANT) at the OMM known form an IMM/OMM contact sites (113;114). The number of contact sites increase with hormone treatment and it is proposed that this increase plays a role in steroidogenesis by limiting the space cholesterol has to travel between the OMM and IMM (187).

TSPO has been also shown to interact with the Golgi protein PAP7 which rapidly moves to mitochondria following hormone treatment of the cells (138). PAP7 acts as an A-kinase anchoring protein (132;133) bringing PKARIα, RIIα and to a lesser extend RIIβ, but not RIβ, to mitochondria (133;188). Though, PAP7’s role is critical for steroid formation, as shown in knock down studies, the evidence accumulated suggest a role in regulating cholesterol movement to OMM but no role in the transfer of cholesterol to the IMM has been identified (132).

In a recent study, we evaluated the mechanism mediating TSPO import into mitochondria (189). TSPO lacks a classical mitochondrial targeting signal sequence. Using site directed mutagenesis, confocal microscopy, Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE), and mass spectrometry (MS) we demonstrated that the initial targeting of TSPO to mitochondria is dependent upon the presence of cytosolic chaperones interacting with the import receptor Tom70. The C-terminus
plays an important role in targeting TSPO to mitochondria whereas its import into the OMM is dependent upon the presence of the Schellman motif. Final integration of TSPO into the OMM occurs via its interaction with Metaxin 1 (189). TSPO import into steroidogenic cell mitochondria is regulated by cAMP (189). Interestingly, in these studies that TSPO initially enters OMM at a 66 kDa protein complex. However, at the end the biologically functional population of TSPO in the OMM is found in a 800 kDa complex (189). MS analysis of the 800 kDa complex identified the isoforms of VDAC and ANT (189).

Using the hormone responsive steroidogenic MA-10 mouse tumor Leydig cells we evaluated the hormonal dependence of the protein composition of the mitochondrial complexes, their ability to import cholesterol and serve as self contained independent steroidogenic units forming pregnenolone. The results obtained suggest that acute hormone-induced steroidogenesis begins at the 800 kDa mitochondrial OMM/IMM contact site complex.
Material and Methods

Materials: Antibodies specific for TOM22 (Sigma), VDAC, Cox IV, Tom40 (Abcam; Cambridge, MA), Metaxin 1 (BD Bioscience; San Jose, CA) GapDH (Trevigen, Gaithersburg, MD) were purchased from the various vendors. StAR antibody was a generous gift from Dr. DB Hales (170). The TSPO polyclonal antibody was developed as previously described (96).

Cell Culture: MA-10 Leydig cells were a gift from Mario Ascoli (University of Iowa, Ames) and were maintained in DMEM/Ham’s F12 (50:50) supplemented with 5% fetal bovine serum (FBS) and 2.5% horse serum at 37 °C and 3.7% CO₂.

CYP11A1 Probe: The fluorogenic probe 22-phenoxazonoxy-5-cholene-3-beta-ol was synthesized in a four step chemical synthesis (US patent 5,208,332 with modifications). 1g of 3-beta-acetoxy-23, 24-bisnor-5-cholenic acid (compound 1) (Steraloids, Inc., Wilton, NH) was dissolved in 5mL of methylene chloride cooled to 0-5°C. 900 mg of tert-butoxy Thionyl chloride was added to the mixture and stirred for 20 h. After a liquid/liquid extraction using NaCl solution, the organic phase was dried. 2.3 mL of a 1.0 M tri-tert-butoxyaluminhydride in tetrahydrofuran was added under argon and cooled to -65°C. 1 h after addition, the mixture was extracted with NaCl/HCl solution. The dry organic phase contains Compound I, 3-beta-acetoxy-5-cholene-22-ol, purified on a silica chromatography column. 175 mg of p-toluenesulfonyl chloride was added to Compound I dissolved in pyridine. The mixture was stirred for 20 h at room temperature. 3-beta-acetoxy-22-p-
toluenesulfonyl-5-cholene (compound II) was obtained after a liquid/liquid extraction diethyl ether/HCl,NaCl. 200 mg of resorufin was added to Compound II in DMSO and stirred under argon at 55°C for 10 days. 3-beta-acetoxy-22-phenoxazonoxy-5-cholene (compound III) was obtained purified by silica chromatography column as small orange needles. Compound III was suspended in 5% KOH/methanol containing 1% water and refluxed for 30 min under argon. After washing the mixture with HCl/water, fluorogenic probe 22-phenoxazonoxy-5-cholene-3-beta-ol (compound IV) was obtained as an orange solide crystallized in methylene chloride/hexane. After synthesis, the sample was dissolved in 10mg/mL ethanol, sonicated and filtered through a .22µm filter. Concentration was determined via spectrophotometer and 1:200 sample was added to the isolated mitochondria or cells.

**Mitochondria Isolation:** Mitochondria were isolated by differential centrifugation as previously described (94). Briefly, HeLa cells or confluent MA-10 cells were washed twice with PBS, harvested in Buffer A (10mM Hepes-KOH, pH 7.5, .2 M Mannitol, .07 M Sucrose, 1mM EDTA, 1x Complete Protease Inhibitor Cocktail Tablet (Roche, Switzerland)) using a cell lifter, and centrifuged at 500 x g for 10 minutes. The cell pellet was resuspended in 5 volumes of Buffer A, incubated at 4°C for 10 minutes and then centrifuged at 500 x g for 10 minutes. The cell pellet was resuspended in 5 volumes Buffer B (40 mM Hepes-KOH, pH 7.5, 500mM sucrose, 160 mM Potassium Acetate and 10mM Magnesium Acetate, 1x Complete Protease
Inhibitor Cocktail Tablets) and sonicated for 10 seconds. Once complete, cells were centrifuged at $500 \times g$ for 10 min. The supernatant was pooled and centrifuged at $10,000 \times g$ for 10 minutes at 4 °C to form a mitochondrial pellet. The mitochondrial pellet was resuspended in 1mL Buffer B and centrifuged at $10,000 \times g$ for 10 minutes to enrich mitochondrial purity; this step was repeated until desired purity was achieved. Once complete, the mitochondria were resuspended in either mitochondria import buffer (3% BSA, 250 mM sucrose, 5 mM MgCl$_2$, 80 mM KCl, 10 mM MOPS-KOH, pH 7.2, 5 mM ADP, and 10mM succinate (Sigma, St. Louis), 1x Complete Protease Inhibitor Cocktail Tablets) to give a final concentration of 1 mg/mL mitochondria for BN-Page and isolated steroidogenic assays.

Blue Native (BN)-PAGE: BN-PAGE was performed as described by Simpson (190). BN-PAGE loading dye (5% w/v Coomassie Brilliant Blue G-250, 500 mM ε-amino-n-caproic acid, and 160 mM BisTris, pH 7.0) was added to the sample supernatants, loaded onto a 4-16% native gel (Invitrogen), and run at 130 V. Once complete, the gel was transferred using a semi-dry transfer apparatus for one hour at 25 V. The PVDF membrane was then fixed and dried. If necessary the membrane was exposed to a multipurpose phosphor screen for 1-7 days and analyzed by phosphorimaging using the Cyclone Storage Phosphor System (Packard BioScience; Waltham, MA). Once complete, the membrane was then used for immunodetection. Image analysis was performed by Multi Gauge V3.0 from FujiFilm.
*Two Dimensional (2D)-SDS-PAGE:* A lane from the one-dimensional, BN-PAGE gel was removed and incubated with reducing solution (50 mM dithiothreitol and 1× NuPAGE LDS Sample Buffer, Invitrogen) for 30 minutes. The solution was removed and replaced with Alkylating Solution (Invitrogen) for 30 minutes prior to the addition of Quenching Solution (Invitrogen) for 15 minutes. The gel strip was placed on the top of a new gel for SDS-PAGE, overlaid with NuPAGE LDS Sample Buffer, and run at 120 V. The gel was transferred and analyzed as described above for BN-PAGE.

*Steroidogenesis assays.* Steroid producing assays were performed by incubating either mitochondria or BN-PAGE spots in Steroidogenic buffer (250mM Sucrose, 10mM Potassium Phosphate Buffer, pH 7.0, 15mM Triethanolamine-HCl, 20mM KCl, 5mM MgCl₂, 5µM Trilostane), initiating steroidogenesis by adding 1/10 volume of Buffer C (150mM Isocitrate, 5mM NADP) to the mitochondria for stated time points (191). The CYP11A1 probe was incubated with the mitochondria or gel spots before the addition of Buffer C for stated time points. Mitochondria were kept on ice until use for no longer than 1 hour. MA-10 cells stimulated with 1mM 8-Bromo cAMP were treated for two hours prior to isolation (Biomol, Farmingdale, NY).

*Photo-cholesterol:* 5 µCi (83.5pmol) of 7-azi-5α-[3,5,6-³H] cholestan-3β-ol ([³H]photocholesterol, American Radiolabeled Chemicals Inc. (St. Louis, MO) incubated with 50 ug of mitochondria isolated from control or cells treated with
1mM of dibutyryl cAMP for thirty minutes. Half of the sample was cross-linked with UVP 3UV Ultraviolet Lamp (Pierce) at (365nm, 8 Watts) for 16 minutes, as determined by the half-life of the photocholesterol, on ice 30mm from the source to the sample. The other half of the sample was placed in the dark. Both samples were solubilized with digitonin and run on a BN-PAGE gel. Samples were transferred to a PVDF membrane and exposed to a phosphoimager screen for 1-3 days.

In-gel Digestion and MS Analysis: Selected protein spots from MA-10 mitochondria extracts separated by BN-Page followed by 2D-SDS-PAGE were subjected to an in-gel digestion procedure as described elsewhere (189). Resulting in-gel digests were desalted using C18 Zip Tips (Millipore) before analysis by nano-flow reversed-phase liquid chromatography (nanoRPLC) using an Agilent 1100 LC system (Agilent Technologies, Inc., Paolo Alto, CA) coupled online to a linear ion trap (LIT) mass spectrometer (LTQ, Thermo Scientific, San Jose, CA). Reversed-phase separations were performed using 75 µm i.d. × 360 µm o.d. × 10 cm long capillary columns (Polymeric Technologies Inc., Phoenix, AZ) that were slurry packed in-house with a 5-µm, 300 Å pore size Jupiter C-18 silica bonded stationary phase (Phenomenex Torrance, CA). After being injected with 5 µl of sample, the column was washed for 20 minutes with 98% solvent A (0.1% formic acid in water, vol/vol) and peptides were eluted using a linear gradient of 2% solvent B (0.1% formic acid in 100% acetonitrile, vol/vol) to 85% solvent B for 110 minutes at a constant flow rate of 250 nl/min. The LIT-MS was operated in a data-dependent mode in which each full MS
scan was followed by seven MS/MS scans where the most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 36%. The temperature of the heated capillary and electrospray voltage (applied on column base) was 180 °C and 1.7 kV, respectively. The CID spectra were searched against a non-redundant human protein database using SEQUEST (Thermo Scientific, San Jose, CA) and results were tabulated for each identified peptide/protein.
Results:

BN-PAGE separation and identification of proteins involved in steroid production:

MA-10 mouse Leydig cells were treated with 50ng/mL hCG for two hours. After
treatment, cells treated and untreated were harvested and the mitochondria isolated
and solubilized with digitonin. BN-PAGE gels were run at 4°C over night,
transferred to membranes and probed for proteins known to play a role in
steroidogenesis. Blotting against TSPO identified a large complex at 66 kDa and a
higher molecular weight complex at 800 kDa; a slight increase in the 800 kDa
complex was observed upon hCG treatment (Fig. 14A). CYP11A1 was show to form
a complex at 140 kDa with no initial change seen between the control and
mitochondria from hCG-treated cells, also present at this complex was ANT (Fig.
14A). VDAC showed a laddering effect at high molecular weights from 240 kDa to
800 kDa. CoxIV, mitochondrial cytochrome C subunit, was present around 440 kDa.
Adrenodoxin reductase was present around 50 kDa (Fig. 14A).

2D-SDS-PAGE analysis of protein complexes separated by BN-PAGE: Upon
completion of the BN-PAGE gel, a lane of gel was removed and proteins were
separated by 2D-SDS-PAGE to further identify protein changes in mitochondria
from control and hCG-treated MA-10 cells. At 66 kDa TSPO was identified to only
be present at the 18 kDa monomeric form, while at 800 kDa it was present only in
polymeric forms (Fig 14B). This suggests that monomeric TSPO present in the
mitochondria runs at 66 kDa while the polymer is primarily present at 800 kDa. A
shift in CYP11A1 (Fig. 14C) and VDAC (Fig 14E) from the lower to higher molecular weights complexes was seen following hCG treatment of the cells and compared to control. Mitochondrial HSP60 immunoreactivity also showed an increase in the hCG-treated samples as well (Fig. 14D).

*Identification of protein complexes through MS:* The 66, 140 and 800 kDa protein complexes identified by BN-PAGE and 2D-SDS-PAGE followed by immunoblot analysis to contain proteins involved in steroidogenesis were further analyzed by MS. Table 3 shows a list of proteins identified in each complex and the number of times they are identified in each sample from the mitochondria isolated from control and hormone treated MA-10 Leydig cells (Table 3).

*Cholesterol is bound by the 66 kDa complex:* To determine where cholesterol could be bound on the mitochondria, azido-[³H]photocholesterol, was added to isolated mitochondria and incubated for 30 minutes. The isolated mitochondria were crosslinked with UV light or for control, left in a dark area. The samples were then centrifuged and solubilized with digitonin and run on a BN-PAGE. As the cholesterol molecules are chemically cross-linked to the interacting protein this cholesterol should not be displaced by the digitonin. The results showed that only the 66 kDa band, where TSPO is located, bound the cross-linked cholesterol (Fig. 15). It is also weakly present on the non-crosslinked samples; as this demonstrates the efficiency of the TSPO monomer in binding cholesterol. Fig 15 also shows that when mitochondria from hormone treated cells were used the amount of crosslinked
cholesterol was reduced, possibly due to isotopic dilution from endogenous steroidogenic cholesterol.

Cholesterol-resorufin functions as a marker for CYP11A1 activity: CYP11A1 was found in the BN-PAGE isolated complexes together with adrenodoxin reductase. To determine whether CYP11A1 in these complexes is active and whether these complexes can function as independent steroidogenic units we devised a method to measure CYP11A1 activity. Cholesterol was conjugated to resorufin dye which upon cleavage it becomes fluorescent with measurable activity at 595nm (Fig. 16A). The cholesterol-resorufin probe was found to work well with MA-10 cells (Fig. 16B) where after incubation for 24 hours with the probe stimulation with hCG resulted in a time-dependent increase in side chain cleavage of cholesterol, marker of probe availability at CYP11A1. The effect was fast with a significant specific induction less than 10 minutes upon addition of the hormone. Upon incubation of the probe with isolated mitochondria a dramatic peak was seen 20 min upon addition of the pore (Fig. 16C).

The BN-PAGE complexes at 66,140, 440 and 800 kDa from control and hormone treated samples were removed from the gel and incubated in steroidogenic buffer containing NADP, ATP, isocitrate and 10uL of resorufin-cholesterol in 200uL. The 66 and 440 kDa complexes showed no CYP11A1 activity under control or hCG treatment. Analysis of the 140 kDa gel spot, where CYP11A1 was shown to be primarily concentrated in controls, indicated a time-dependent CYP11A1 activity.
(Fig. 17A). However, this activity was lost in the mitochondria from hCG-treated cells (Fig. 17B). In the 800 kDa complex, control samples demonstrated no CYP11A1 activity (Fig. 16A) whereas upon hCG treatment a strong CYP11A1 activity was observed (Fig. 17B). The later figure clearly shows that there is shift of the CYP11A1 activity from the 140 kDa complex to the 800 kDa complex.
Discussion:

BN-PAGE is a technique that allows the separation of protein-protein complexes based solely on size; the use of digitonin functions by binding to cholesterol and precipitating; creating holes in the membrane (192). This technique has been shown to allow protein complexes to remain active in the gel due to the weak solubilization that still leaves protein-lipid interactions intact (193). We used this method to answer many questions in steroidogenesis where lipid-protein and protein-protein interactions are the regulating mechanisms. Specifically, it allows the identity of the entry site of cholesterol into mitochondria as well as the site where it is metabolized.

In previous experiments, we demonstrated the formation of a hormone-dependant protein complex in the mitochondria conferring the ability to import cholesterol into mitochondria and form steroids (138). Through BN-PAGE membrane analysis we showed that TSPO forms complexes at both 66 kDa and 800 kDa molecular sizes and further 2D-SDS-PAGE analysis demonstrated that the 66 kDa complex contained only TSPO monomers whereas the 800 kDa complex contained polymers. Upon treatment of Leydig cells with hCG a shift was seen in TSPO from monomers at 66 kDa to polymers at 800 kDa, confirming the observation the that TSPO polymerization occurs after exposure to hormones (104;107) . This change may reflect the previous reported hormone-induced changes in radioligand binding characteristics of TSPO (194). Based on these data we proposed that the 800 kDa
complex is the site of the functional TSPO (189) and the site of cholesterol transfer from OMM to IMM site function in the transfer of cholesterol to the IMM for steroidogenesis.

Detailed analysis by MS and immunoblotting identified a number of proteins in the 140 and 800 kDa complexes including VDAC, ANT, CYP11A1, Tim44 and Tim50 as well as other proteins that belong both to OMM and IMM. These data suggest that these complexes may represent OMM/IMM contact sites, the place that has been postulated for years to be the site where steroidogenic cholesterol crosses from OMM to IMM bypassing the aqueous intermembrane space and loaded onto CYP11A1. In mitochondria from hormone-treated Leydig cells CYP11A1 migrated towards the higher molecular weight complex and the same was true for most of the proteins examined. These results suggest that upon hormone treatment the 800 kDa complex becomes the major complex, containing a functional TSPO and high levels of CYP11A1. Interestingly, the 78 kDa glucose-regulated protein precursor (GRP 78) was also identified in the 800 kDa complex. This protein is the also precursor of the steroidogenesis activating polypeptide shown to activate adrenal steroidogenesis (195).

Despite the four decades-long efforts to identify the mechanisms underlying the hormonal-induction of cholesterol transport into mitochondria, there is no information about the actual site of cholesterol entry into these organelles. It is clear that this site must be in an environment allowing for its segregation from the
structural cholesterol of the OMM. In an effort to identify this site, mitochondria were isolated from MA-10 cells, incubated with the photoactivatable \(^3\)H-
photocholesterol, and exposed to UV. Proteins separated by BN-PAGE migrating at 66 kDa bound specifically cholesterol. Immunobloting demonstrated the co-localization of the cholesterol binding site with TSPO at the 66 kDa complex where TSPO enters into mitochondria (189). Mitochondria from hormone-treated cells showed less photocholesterol binding probably due to isotopic dilution by the endogenous cholesterol accumulating in the site. We have proposed that TSPO segregates the steroidogenic pool of cholesterol from the structural cholesterol present in OMM (131). Hormonal stimulation of the cells results in TSPO polymerization move to the 800 kDa complex whereas in the same time the affinity for cholesterol falls (107) so that cholesterol can now become available for steroidogenesis.

To examine whether the 800 kDa complex is functional in steroidogenesis we set up a system to measure \textit{in-vitro} BN-PAGE and in test tube as well as in cells CYP11A1 activity. In 1990, Marrone et al. reported the synthesis of a cholesterol-resorufin probe with activity in isolated mitochondria and cells (196;197). This probe was not available and we synthesized a new probe following the basic steps outlined by the authors (US patent 5,208,332) with some modifications. Incubation of cholesterol-resorufin with the gel spot together with the necessary cofactors NADP, isocitrate and ADP resulted in the oxidation of the cholesterol-resorufin
molecule by CYP11A1 producing pregnenolone and resorufin. Considering the minute amounts of enzyme and materials available within the gel spot it would have been impossible to measure pregnenolone or isocaproic acid levels to determine CYP11A1 activity without the help of the resorufin fluorescent dye. The ability of the 140 kDa complex to produce steroids was lost upon hCG stimulation, suggesting the enzyme or protein necessary to oxidize cholesterol were no longer present or present in its active form. Instead the 800 kDa mitochondrial complex from hCG treated gained the ability to rapidly metabolize the CYP11A1, suggesting the formation of a larger functional molecular weight complex in the mitochondria upon hCG stimulation. None of the 66 kDa, 440 kDa mitochondrial complexes isolated from control and hCG-treated cells were able to metabolize the cholesterol-resorufin probe.

Once cholesterol is translocated to the IMM, its conversion to pregnenolone occurs with CYP11A1 but also with the auxiliary electron transferring proteins, adrenodoxin and adrenodoxin reductase. These proteins function in the transfer of electrons from NADPH to the substrate (198). A ternary complex of adrenodoxin (Adx), adrenodoxin reductase (AdR), and CYP11A1 has been shown to form in the presence of cholesterol and phospholipids (199). Immunoblot analysis indicated that adrenodoxin reductase was not associated with the BN-PAGE complexes, although AdR was found in both the 140 and 800 kDa complexes by MS. A possible reason
AdR was not observed by immunoblot analysis the epitope was masked due to complex formation in the 140 and 800 kDa complexes.

Because many IMM proteins were identified by MS at the 140 kDa complex, such as Tim44 and Tim50 with adrenodoxin reductase, CYP11A1, ANT, and others, we propose that this protein complex is primarily located in the IMM. This observation would support the findings that the CYP11A1 is active in the IMM (1)

Upon solubilization of the mitochondria, analysis by BN-PAGE exposes this basal complex. Control cells also have an 800 kDa complex but with minimal function. Following hCG treatment IMM/OMM contact sites are formed and many proteins move into the 800 kDa complex. MS analysis of the 800 kDa complex identified proteins and enzymes identified known to be present at both the ER and Golgi apparatus, suggesting a mechanism that would allow a further increase of cholesterol targeted to the OMM and transferred to the IMM through this newly formed OMM/IMM contact sites. We propose that the formation of an intra-mitochondrial contact sites form following hormonal stimulation as previously suggested by Stevens et al. (187). This contact sites may also contain proteins present at the established Mitochondrial-ER contact site (MAM) and the Golgi apparatus which in turn would bring cholesterol and proteins that could function in cholesterol transfer at the OMM and steroidogenesis.
SUMMARY AND CONCLUSIONS

The first metabolic step of steroidogenesis occurs at the inner mitochondrial membrane (IMM), where the cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone (1). The transfer of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in hormone-induced steroid formation. To ensure that this step is achieved efficiently; free cholesterol present at the outer mitochondrial membrane is transferred to the inner membrane. This is accomplished through a series of steps that involve various intracellular organelles and proteins such as the translocator protein (18 kDa, TSPO) and steroidogenic acute regulatory (StAR) protein. TSPO is a high-affinity drug- and cholesterol-binding mitochondrial protein. StAR is a hormone-induced mitochondria-targeted protein that has been shown to promote cholesterol transfer into mitochondria. Through the assistance of proteins, such as the cAMP-dependent protein kinase regulatory subunit Iα (PKA-R1α) and TSPO-associated protein, PAP7, cholesterol is transferred to and docked at the outer mitochondrial membrane. The association of TSPO with the outer/inner mitochondrial membrane contact sites drives the intramitochondrial cholesterol transfer and subsequent steroid formation. The hypothesis examined in this thesis is that delivery of cholesterol into the mitochondria, and thus steroidogenesis, is driven by a series of protein-protein interactions.
Initial studies with CYP11A1 proposed that this enzyme, which is responsible for cleaving the cholesterol side chain, was the rate limiting step in the production of steroids. This observation was shown to be incorrect as upon the addition of cycloheximide, a protein synthesis inhibitor, steroidogenesis was shown to be inhibited in the adrenals, though cholesterol was able to be loaded onto the OMM (70). These results suggested that the ability of cholesterol to be transferred from the OMM to the IMM is the primary control step in the production of steroids and therefore necessary for the activity of CYP11A1 (200). Further experiments were performed using a membrane permeable cholesterol analog, 22R-hydroxycholesterol, which can bind to the active site of CYP11A1 without having to being translocated across the OMM and was able to be metabolized to pregnenolone (201). This suggested that the activity of CYP11A1 was not the rate limiting step in steroidogenesis, but that the transfer of cholesterol to IMM is the rate limiting step of steroidogenesis with CYP11A1’s activity being dependant upon the ability of cholesterol to be transferred to the IMM. These findings resulted in the focus of steroid production shifting from the study of CYP11A1 to identification proteins that function in cholesterol transport to the mitochondria resulting in steroid formation.

In 1977, TSPO was initially identified in the kidney and later found to be present at high concentrations in steroidogenic cells (5). As it was shown to be present primarily in the OMM and have high cholesterol binding affinity, it was suggested it could play a role in steroidogenesis by assisting with the translocation of
cholesterol from the OMM to the IMM (94). Its role in steroidogenesis was confirmed after decreasing TSPO protein expression in mouse Leydig cells, resulted in reduced steroid production as compared to control; these findings were further confirmed in adrenal cells (102). These experiments demonstrated the importance of TSPO in cholesterol transfer to the IMM, providing a mechanism for cholesterol transport that was located on the OMM to the IMM.

Studies have demonstrated that only 20-40% of adrenal mitochondrial cholesterol is depleted upon initiation of steroidogenesis, suggesting there is a mitochondrial pool of cholesterol present for steroidogenesis which is utilized upon hormonal stimulation (202). As this mitochondrial pool of cholesterol is utilized, more cholesterol must then be targeted to the mitochondria for steroidogenesis. The previous results demonstrated that cycloheximide, which inhibits protein synthesis, also inhibited steroidogenesis implying there was a “labile protein factor” that played a role in steroid production. It was suggested that this protein bound the cholesterol needed for steroidogenesis to continue, interacting with the mitochondria upon tropic hormone activation and assisting with the transfer into the IMM. In 1994, a 32-kDa protein whose expression correlated with the initiation of steroid synthesis was identified, cloned and named the Steroidogenic Acute Regulatory protein (StAR) (74). StAR was shown to contain both an N-terminus mitochondrial targeting sequence and a C-terminus cholesterol binding domain, suggesting a mechanism for which it could function in targeting cholesterol to the mitochondria (76;78). Initial
studies on the mature 30-kDa form of StAR showed that it is necessary for steroidogenesis and upon reduction in cAMP-dependant StAR expression, steroidogenesis was inhibited (124). It was further shown however, that it was not the 30-kDa form, but rather the precursor 37-kDa protein that was important for steroidogenesis and it was acting outside the OMM (79). These results, which identified the mechanism of StAR’s action at the OMM, laid the foundation for the role of protein-protein interactions in steroidogenesis by providing the linking factor for cholesterol transport from the cytosol to the mitochondria.

To determine then if StAR played a role in transferring cholesterol to TSPO for import into the mitochondria several studies were performed and no physical interactions between TSPO and StAR could be identified (123). A functional interaction was shown, however, through antisense knockdown of either StAR or TSPO; each protein was necessary for steroidogenesis, and TSPO was found to be necessary for mitochondrial processing of the StAR protein (124). These observations led to the proposal that other proteins must function in cholesterol transport to the OMM and in steroidogenesis. Through yeast two-hybrid screens it was identified that TSPO interacts with PBR associated protein 7, PAP7; moreover, PAP7 was additionally shown to interact with PKA-R1α, a cAMP-dependant protein kinase. The identification of these two proteins provided the foundation for a hypothesis that a scaffolding complex forms at the OMM and is necessary for steroidogenesis (133). While this scaffolding complex was shown to interact
through yeast two hybrid assays and confocal microscopy, no physical interactions could be identified to confirm its presence at the OMM.

Chapter 1 of this thesis addressed the question of a mitochondrial scaffolding complex. We focused upon demonstrating the formation of the protein complex of TSPO, StAR, PAP7 and PKA-RIα, called the transduceosome, at the OMM. Through the use of photoactivatable amino acids, the presence of the transduceosome at the OMM, was confirmed. This was seen after incubation of the photoactivatable amino acid and crosslinking; western blot analysis identified TSPO, StAR, VDAC, PAP7 and PKA-RIα all present at a high molecular weight complex. As StAR requires phosphorylation for its activity at the OMM, the transduceosome brings StAR in close proximity PKA-RIα for phosphorylation.

Upon incubation of radiolabeled cholesterol in the media with MA-10 cells, it was further identified that cholesterol was also present in the transduceosome complex. This provided another mechanism of action in the trafficking of cholesterol to the mitochondria for use in steroidogenesis. The identification of this novel mechanism shifted the assumption held previously that cholesterol transported from intracellular stores into mitochondria is mediated by a single cholesterol transport protein, StAR but now to the presence of a multi-protein scaffolding complex, the transduceosome. This proposal lays the foundation for a novel mechanism of cholesterol delivery to the mitochondria, both for the basal and hormonally-stimulated cells, as this complex was also identified at the basal state on
the OMM. As the tranduceosome could be utilized for the targeting of non-
steroidogenic cholesterol to the mitochondria, the basal state could also provide the 
scaffolding mechanism for the amplification of cholesterol transport upon hormone 
stimulation for StAR to act upon. Through the tranduceosome, StAR could amplify 
the basal state of cholesterol transfer in to the mitochondria increasing the amount of 
cholesterol available for transfer CYP11A1. Further experiments could analyze the 
role of cholesterol in this complex; specifically the mechanisms by which it is 
transferred.

As the tranduceosome protein complex is anchored around the OMM protein 
TSPO, in chapter 2, we wanted to determine the protein-protein interactions that are 
necessary for the import of TSPO into the OMM. Cytosolic proteins, Heat Shock 
Protein (HSP) 70 and 90, were found to function in the targeting of TSPO to the 
mitochondria. Also necessary for TSPO targeting to the OMM were the C-terminus 
amino acids #151-169 and amino acids #103-109. Amino acids 103-109 form a 
Schellman motif, which functions in maintaining the correct morphology for TSPO 
import and protein-protein interactions. Upon targeting of TSPO to the 
mitochondria, the HSP’s interact with the Translocase of Outer Mitochondrial 
protein 70, TOM70. TSPO is then transferred to OMM protein Metaxin1 in an ATP-
dependant manner. Metaxin1 assists with TSPO’s final integration into the 
membrane; this complex has been shown to form a 66kDa on a BN-PAGE. The rate
of import of TSPO at the 66kDa was shown to be increased upon hormone stimulation, suggesting TSPO’s import is regulated by hormonal stimulation.

The data gathered herein provides a novel pathway for the insertion of multi-spanning, alpha helical OMM proteins into the mitochondria, as previously no pathway had yet been identified. Protein-protein interactions between the TOM and the SAM protein complexes is a common pathway used for β-barrel protein insertion, though it was unknown if these two protein complexes are also used for α-helical protein insertion. The identification of the use of the receptor from the TOM complex, TOM70, and a protein from the SAM complex, Metaxin1, suggests there are protein-protein interactions between the two complexes that are used for the membrane insertion of α-helical proteins. Therefore, this pathway for protein insertion maybe more common or function in the import of more OMM proteins than previously presumed (164).

Further study of the import of TSPO demonstrated that its import was increased with hormonally stimulated isolated mitochondria as compared to control. Moreover, the amounts of lipids in the mitochondria are altered in response to hormonal stimulation; for example the amounts of cardiolipin and cholesterol are increased, which could alter the rate of TSPO import. It has been suggested that the import of mitochondrial proteins alters the regulation of mitochondrial lipids (203). Therefore it would be interesting to know, if increased import would occur with all mitochondrial proteins or only OMM proteins like TSPO. It has also been shown
that the presence of cardiolipin can stabilize protein import complexes located both at the IMM and OMM, increasing the ability of TSPO to be imported into OMM through the stabilization of the TOM or the SAM complex (204). Beside cardiolipin, cholesterol levels in the OMM and IMM are increased upon hormonal stimulation; it is unknown what effect this could have on protein-protein interactions necessary for TSPO import, as cholesterol is known to decrease membrane fluidity and alter membrane permeability. To further understanding of this event, it will be important to see the rate of change of lipids present in the OMM, specifically cholesterol and cardiolipin. If these lipids are altered, it would be interesting to determine what decreasing the concentrations of cardiolipin or cholesterol would alter the import of TSPO.

Once TSPO has been successfully imported into the mitochondria, as shown in chapter 2, it interacts with other proteins present in the OMM. In chapter 3, we continued the study of TSPO’s protein-protein interactions occurring in MA-10 cells treated with hCG. This allowed us to identify protein-protein interactions that were occurring in a hormone-dependent manner as compared to control. TSPO was identified in a BN-PAGE gel at 66kDa in a monomer form and at 800kDa in polymer form. As polymerization of TSPO occurs upon hormonal stimulation (107), correlating with steroid synthesis, this finding suggested that the 800kDa complex may be functional in steroidogenesis. Immuno blotting of BN-PAGE gels identified that CYP11A1 was present at 140kDa along with ANT. Upon hCG treatment, it was
shown that CYP11A1 was present at the 140kDa complex and at higher molecular weights upon stimulation up to the 800kDa complex. To examine whether these complexes contained CYP11A1 activity we used a chemically-modified cholesterol, resorufin, which upon cleavage by CYP11A1 released a fluorescent probe and pregnenolone. These experiments demonstrated that 140kDa basal complex was capable of producing steroids while the 140kDa complex that had been exposed to hCG was not. The reverse was seen of the 800kDa complex with the basal complex was not capable of producing steroids while the hCG 800kDa was, suggesting a shift in proteins necessary for steroidogenesis from 140kDa to the 800kDa complex.

The data gathered from both the BN-PAGE western blot analysis and the resorufin dye identifies of a protein complex present in the mitochondria that has been shown to be functional in steroid production. As previously mentioned, the rate limiting step in the production of steroid hormones is the transfer of cholesterol from the OMM to the IMM, with functional CYP11A1 located at the IMM and segregated from the cholesterol. The identification of functional CYP11A1 present at 140kDa on BN-PAGE interacting with another IMM protein, ANT, suggests this is the complex that present at basal state. Upon addition of the cholesterol in the gel spot at 140kDa basal state the complex is capable of producing pregnenolone. Upon hormonal stimulation the steroidogenic complex is not present at 140kDa but shifts to 800kDa, where CYP11A1 can now interacts with OMM proteins, VDAC and TSPO, both proteins present in the transduceosome and components of mitochondrial
contact sites (110). It is the presence of the OMM contacting the IMM would allow for easy transfer of cholesterol to the IMM and interaction with CYP11A1.

Contact sites are dynamic localization of where OMM and IMM meet and have previously been proposed to function in cholesterol transport to CYP11A1 in the IMM (205). The formation of a contact site would allow the direct transfer of cholesterol from one membrane to another while removing the inner mitochondrial membrane space the hydrophobic cholesterol would have to cross. Contact sites between VDAC and ANT have previously been proposed to control rates of apoptosis and energy metabolism, in which these cellular functions can have several affects on steroid synthesis (206). This work demonstrates the formation of contact sites in the mitochondria for steroidogenesis as confirmed through both mass spectrometry and western blot analysis. The lack of this contact site as seen from control mitochondrial as compared to hormonally treated mitochondrial demonstrates how important these intramitochondrial formations are for steroid synthesis.

Further analysis of these contact sites can be used to identify the lipid composition necessary for steroid formation. For example, the amount of cardioplin, which has already been implicated for TSPO import, has also been shown to be important in CYP11A1:adrenodoxin complex formation and function (207). Further analysis through mass spectrometry can also be used to determine which enzymes and proteins are necessary for the generation of steroids, specifically
assisting with the formation of NADPH for the reduction of cholesterol and to identify which other protein complexes can help function in contact site formation. The results in this thesis confirm and extend our understanding of the importance of protein-protein interactions in steroidogenesis from mitochondrial import, cholesterol transport and steroid biosynthesis. Mitochondrial protein import of TSPO was shown to be dependant upon the novel pathway of protein interactions of heat shock proteins with mitochondrial protein import receptor TOM70 and Metaxin 1. Its import helps sustain the role TSPO plays in the initiation and maintenance of steroidogenesis at the OMM and cholesterol transport into the IMM. This ability of cholesterol transport was demonstrated to be dependant upon the formation of a hormone-induced protein complex present between the inner and outer mitochondrial membrane consisting of TSPO, VDAC, ANT and CYP11A1. The supply of cholesterol to the mitochondria for steroidogenesis is maintained by a signaling mechanism between TSPO, StAR, VDAC, PAP7 and PKA-RIα, functioning in the transport of cholesterol to the mitochondria from the cytosol. The identification and confirmation of these protein-protein interactions further confirm their important role in the cell for steroidogenesis; from the cytosol to the outer to the inner mitochondrial membrane and finally to CYP11A1 and steroid production.
Figure 1. Trafficking of cholesterol to the mitochondria for steroidogenesis.

Pathway 1: Cholesterol synthesized in the ER is trafficked to the Golgi apparatus where it can be targeted to the mitochondria via PAP7 protein for steroidogenesis. Passive diffusion from the ER to the mitochondria, shown here with the corresponding cholesterol molecules present on both organelles, is another possible pathway for steroidogenic cholesterol to be transferred to the mitochondria.

Pathway 2: Low density lipoprotein (LDL), containing cholesterol, binds to the LDL receptor and is trafficked through the endosomal pathway. MLN-64 assists with the transfer of cholesterol to the mitochondria from the late endosomes and lysosomes for use in steroidogenesis. NPC1 and NPC2 associate with MLN-64 for cholesterol transfer out of the lysosomes, although it is not known if this cholesterol is used for steroidogenesis. Pathway 3: Cholesterol is transferred from high density lipoprotein (HDL) to the plasma membrane by the SR-BI receptor. Hormone-sensitive lipase (HSL) converts esterified cholesterol from the plasma membrane to free cholesterol, which can be used for steroidogenesis. Pathway 4: HSL also interacts with esterified cholesterol present in the lipid droplets (LD), which converts esterified cholesterol to free cholesterol for use in steroidogenesis as well. Free cholesterol from the LD can interact with lipid-binding proteins present in the cytosol for delivery to the mitochondria.
Figure 1:
Figure 2: A) The structure of acetate (Carbon is grey, Oxygen is red). B) Structure of Isoprene, basic carbon backbone structure used to produce cholesterol. C) Initial steps of cholesterol synthesis with the conversion of Acetyl-CoA to Acetoacetyl-CoA by thiolase, enzymatic addition of each molecule to form HMG-CoA by HMG-CoA synthase and further reduction to mevalonate by HMG-CoA reductase. D) Dimethylallyl pyrophosphate and Isopentyl pyrophosphate (IPP) are interconverted by IPP isomerase with one molecule of each joined head to tail to produce geranyl pyrophosphate. Another molecule of IPP is joined head to tail by prenyl pyrophosphate to the geranyl pyrophosphate to produce farnesyl pyrophosphate. The catalyzing of two farnesyl pyrophosphate in head to head condensation occurs though squalene synthase to produce squalene. E) Squalene is converted to 2,3-Oxidosqualene by squalene epoxidase and further is converted to lanosterol through squalene oxidocyclase. The conversion of lanosterol to cholesterol occurs through 19 steps not demonstrated here.
A. Acetate

B. Isoprene
   (2-methyl-1,3 butadiene)

C. Acetyl-CoA
   \[\text{Acetyl-CoA} \xrightarrow{\text{Thiolase}} \text{Acetoacetyl-CoA}\]

   \[\text{H}_3\text{C} - \text{C} = \text{O} - \text{SH} \quad \text{H}_3\text{C} - \text{C} = \text{O} - \text{CH}_2 - \text{SH}\]

   \[\text{HMG-CoA Synthase}\]

   \[\text{CoA} \quad \text{HMG-CoA}\]

   \[\text{H}_2\text{O} - \text{C} = \text{O} - \text{SH} - \text{CoA}\]

   \[\text{HMG-CoA reductase}\]

   \[\text{Mevalonate}\]

   \[\text{2NADPH} \quad \text{2NADP}^+ \quad \text{CoA}\]
D. Dimethylallyl Pyrophosphate  \[\xrightarrow{Isopentenyl Pyrophosphate isomerase} \text{Isopentyl Pyrophosphate}\]

\[\xrightarrow{PPI} \text{Geranyl Pyrophosphate}\]

\[\xrightarrow{PPI} \text{Farnesyl Pyrophosphate}\]

\[\xrightarrow{\text{Farnesyl pyrophosphate} \text{NADPH} \text{2 PPI}} \text{Squalene Synthase}\]

\[\xrightarrow{\text{NADPH}} \text{Squalene}\]
E.

Squalene + O₂ → 2,3-Oxidosqualene

\[ \text{Squalene epoxidase} \]

\[ \text{Squalene Oxidocyclase} \]

Cholesterol → Lanosterol

19 rxn's
**Figure 3  Cholesterol-binding domains of StAR and TSPO.** A) Human StAR protein modeled with cholesterol (brown) present in the sterol-binding domain (PDB ID code: 2I93). Homology models are deduced via the Swiss-model services under project (optimize) mode using the crystal structures of human MLN64 (PDB ID code: 1EM2) as template ([http://swissmodel.expasy.org/SWISS-MODEL.html](http://swissmodel.expasy.org/SWISS-MODEL.html)). B) Molecular model of TSPO's five alpha helices in the presence of cholesterol, demonstrating a pore accommodating a cholesterol molecule. Homology modeling of mouse TSPO was performed using apolipophorin-III from *Manduca sexta* as template (PDB ID code: 1EQ1) (208). C) Ribbon diagram of mTSPO, showing the five helices as well as the CRAC domain, consisting of Leu/Tyr/Arg residues, and cholesterol (top view). D) Docking model of cholesterol to the TSPO CRAC domain. The accessible surface of the peptide and cholesterol molecules are represented in red and blue, respectively (reprinted with permission from (173)).
Figure 3:
Figure 4: Protein-protein interactions at the OMM. A) Proposed basal-state protein-protein interactions of TSPO, VDAC, and ANT. B) Transduceosome complex formed after acute hormonal stimulation. The TSPO, PAP7 (ACBD3), and VDAC complex recruits PKA-R1α, which is shown binding to PAP7 (ACBD3). The accumulation of cAMP activates PKA-R1α, releasing the catalytic subunits and phosphorylating StAR present at the OMM. StAR interacts with both TSPO and VDAC and is imported into the IMM. Cholesterol is imported into the IMM with the assistance of the transduceosome complex and the presence of DBI; there the cholesterol interacts with the CYP11A1 side chain cleavage enzyme to be converted to pregnenolone. ANT is represented in the IMM as identified from previous isolated complexes with TSPO.
Figure 4:

A) Cytosol

B) Outer
Contact site
Inner
Matrix

Legend:
- Catalytic subunit
- cAMP
- Cholesterol
- Pregnenolone
Figure 5: Photo-crosslinking amino acids in immunoprecipitation: A)

Amino acids used for photo crosslinking B) Transfection of GFP-TSPO and incubation of photoamino acids for 24 hours, cells were cross-linked with UV light, harvested and immunoprecipitated with GFP antibody.
Figure 5:

A.

B.

<table>
<thead>
<tr>
<th>Minutes</th>
<th>No Photo Leucine</th>
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<td>0</td>
<td>10</td>
</tr>
<tr>
<td>hCG treatment</td>
<td>Anti-GFP</td>
<td>Anti-PBR</td>
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</table>

100kDa
**Figure 6: Protein-protein interactions identified by cross-linking.** COS-F2-130 cells transfected with StAR cDNA were cultured with media supplemented with photoactivable amino acids as described under “Experimental Procedures.” Cells were washed and irradiated with UV light, and proteins were analyzed by electrophoresis followed by immunoblotting with anti-TSPO and anti-StAR antisera (top panel). MA-10 cells were cultured with media supplemented with photoactivable amino acids (P-amino acids). At the end of the incubation, cells were treated with 50 ng/ml hCG for the indicated periods of time. Cells were washed and irradiated with UV light as described under “Experimental Procedures.” Cells were collected, and proteins were separated by electrophoresis followed by immunoblotting with anti-TSPO (A, B, and C), anti-StAR (A and C), anti-PAP7, anti-PKARIα, anti-VDAC1, anti-ANT, or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antisera (C). Data (means; S.E. <20% of means) obtained from three independent experiments were analyzed by image analysis. D, MA-10 cells were transfected with pEGFP-Tspo. After 48 h, cells were incubated with \[^3\text{H}]\text{photocholesterol} together with the artificial amino acids and incubated for 24 h. Cells were treated with hCG and irradiated with UV light as described above. At the end of the treatment, cells were harvested, and cell lysates were subjected to immunoprecipitation with anti-GFP antibodies followed by immunoblotting with anti-TSPO antisera. Radioactivity content in the immunoprecipitates was measured by liquid scintillation spectrometry.
Figure 6:
Figure 7: Identification of TSPO import complexes in the OMM. A) Isolated mitochondria from HeLa cells were incubated with in vitro-transcribed/translated $[^{35}\text{S}]$-TSPO. The BN-PAGE gel was transferred to PVDF and exposed to a multipurpose phosphor screen (left panel) or blotted with antisera against TSPO, Tom 40, and Tom22 (right panel). B) In vitro-transcribed/translated TSPO import into mitochondria examined at either 4 °C or 33 °C; at the stated time points, one-half of the import reaction was treated with proteinase K and both samples were analyzed by BN-PAGE. C) In vitro-transcribed/translated $[^{35}\text{S}]$-TSPO and $[^{35}\text{S}]$-Tom70 were incubated with HeLa cell mitochondria and treated with sodium carbonate on ice for 30 minutes. After treatment, solubilized mitochondria were centrifuged and the pellet and supernatant were separated by SDS-PAGE, the membrane was blotted with anti-HSP-60.
Figure 7:
**Figure 8:** TSPO import is dependent upon heat shock proteins and ATP for import.  
A) Effects of C90 incubation with TSPO. TSPO was imported as described above (control).  
B) Effect of ATP depletion, 18μM geldanamycin, and 1mM novobiocin on TSPO import.  
C) Quantification of imported TSPO. Results shown are means±sem from three independent experiments.  
D) Nickel-sepharose pull-down assay of 35S-TSPO and 35S-PIC. When indicated geldanamycin or novobiocin were added to the reaction mixture.
Figure 8:
Figure 9: Identification of the TSPO amino acid sequence(s) responsible for targeting the protein to the OMM. MA-10 cells were transfected with either wild-type GFP-TSPO or various constructs of GFP-TSPO, stained with Mitotracker CMX and visualized by confocal microscopy. Each panel shows the construct used, the GFP staining, the mitochondrial staining, and the merged image. A) Wild-type TSPO with GFP linked to the N-terminus. Cells were also transfected with GFP-Δ1-7-TSPO (B), GFP- Δ1-28-TSPO (C), GFP-Δ1-48-TSPO (D), GFP- Δ1-68-TSPO (E), GFP- Δ1-85-TSPO (F), GFP- Δ1-110-TSPO (G), GFP-Δ157-169-TSPO (H) GFP-Δ151-169-TSPO (I). The cholesterol binding domain, GFP-Δ148-157-TSPO (J), or the Schellman motif, GFP-Δ103-108-TSPO (K) were removed and transfected. Construct GFP-G106A-TSPO (L) point mutation altering the Schellman motif and constructs GFP-Leucine112-114-TSPO (M) and GFP-Leucine137,138,141-TSPO (N) were also transfected as described under experimental methods.
Figure 9:
Figure 10: TSPO protein regions and amino acids required for insertion into the OMM. A) Model of the 18 kDa TSPO protein inserted into the OMM. Blue amino acids represent the C-terminus, red amino acids represent the mutated leucine residues, and green amino acids show the Schellman motif with the yellow amino acid in the middle representing the mutated glycine. B) Schellman motif mutants of the TSPO protein were compared with wild-type TSPO for import into HeLa cells mitochondria, followed by analysis by BN-PAGE. C) Effect of removal of the C-terminus with constructs Δ157-169 and Δ151-169 on inhibition of TSPO import into isolated mitochondria. D) Effect of leucine mutations on in vitro-transcribed/translated radiolabeled TSPO import into HeLa cell mitochondria. E) SDS-PAGE of radiolabeled constructs from import studies for confirmation of size. F) Measurement of progesterone production produced by MA-10 was performed as stated in the methods. Results shown are means ± SEM from three independent experiments (n = 9).
Figure 10:
Figure 11: TSPO import is dependent on Metaxin 1. A) MA-10 mitochondria incubated with in vitro-transcribed/translated TSPO analyzed by BN-PAGE, and blotted with anti-metaxin 1 antibody. B) In vitro-transcribed/translated TSPO incubated with mitochondria isolated from MA-10 cells control, Metaxin 1-depleted, and MA-10 cells treated with scrambled siRNAs. C) Immunoblot analysis for VDAC, mitofilin, TSPO and metaxin1 in mitochondria isolated from MA-10 cells treated with the various siRNAs. D) Quantification of imported TSPO. Results shown are means±sem from three independent experiments.
Figure 11:
Figure 12: TSPO import is increased with cAMP stimulated mitochondria. A) Mitochondria isolated from MA-10 cells incubated with +/- 1mM cAMP were incubated with radiolabeled TSPO, analyzed via BN-PAGE. B) Quantification of imported TSPO, results shown are means±sem from three independent experiments. C) Western blot of MA-10 cell incubated with +/- 1mM cAMP. D) QPCR analysis of MA-10 RNA +/- 1mM cAMP
Figure 12:
Figure 13: TSPO import into the OMM. We have outlined the steps necessary for TSPO targeting and insertion into the OMM. TSPO is targeted to the OMM through its interactions with cytosolic chaperones, Hsc70 and Hsp90. At the OMM, TSPO interacts with Tom70 and is released from the chaperones in an ATP-dependent manner. TSPO insertion into the OMM is dependent upon Metaxin 1 (Mtx1) and TSPO structure, where both the C-terminus and the Schellman motif (amino acids 103-108) are necessary to form a 66 kDa complex. Once import is complete, TSPO associates with other protein complexes found in the OMM identified previously as VDAC and ANT.
Figure 13:
Figure 14: Blue-Native Page Gels identify protein complexes. Mitochondria were isolated from MA-10 cells that were treated or untreated for two hours in the presence of 50ng/mL hCG. Upon isolation, samples were solubilized with 1% digitonin and run on a BN-PAGE gel. Immunoblot analysis identified TSPO at 66kDa and 800kDa, Cyp11A1 at 140kDa, VDAC laddering at 242kDa, 440 kDa, 600kDa and 800kDa., COXIV at 480kDa, and Adrenodoxin Reductase (AdR) present below 66kDa. B) Upon the removal of a lane from the BN-PAGE gel and incubation in SDS, an SDS 2D-PAGE gel was run; identifying TSPO at an 18kDa monomer at 66kDa while at 156 kDa a polymer at the BN-PAGE 800kDa is present. C) Cyp11A1 formed a band across the gel at 60kDa. D) HSP60 was present at 60kDa. E) VDAC is present at 30kDa.
Figure 14:
Figure 15: Lipids present in the identified protein complexes. Isolated mitochondria solubilized with digitonin and run on BN-PAGE gel, control and hCG treated. Radiolabeled cholesterol binding to TSPO both in the presence and absence of cross-linking UV light in also with control and hCG treated mitochondria.
Figure 15: [Image of a gel electrophoresis diagram with bands and annotations for anti-TSPO, HCG, control, cAMP, and molecular weights (M.W.)]
Figure 16: Cyp11A1 probe is active in whole cells and isolated mitochondria.

A) Synthesis of Cyp11A1 Probe B) MA-10 cells were incubated with or without 5uL of the Cyp11A1 probe for 24 hours after of which 1mM 8-bromo cAMP was added to the half the samples. Measurement at 595nm showed an increase in the fluorescence in the cAMP treated cells with the Cyp11A1 probe. C) MA-10 cells control or treated with 1mM 8-bromo-cAMP, after the mitochondria were isolated and incubated with 5uL ethanol, 5uL dye or control
Figure 16:
Figure 17: Cyp11A1 probe shows activity in the BN-PAGE gel. Isolated mitochondria from 50ng/mL hCG treated or untreated MA-10 cells were run on a BN-PAGE gel and spots previously identified to contain proteins necessary for steroidogenesis were isolated. Samples were incubated with steroidogenic import buffer, NADP, and Isocitrate. A) Control samples show fluorescent activity at the 140kDa gel spot while no other samples did. B) hCG treated MA-10 cells showed activity at the 800kDa spot while no other areas showed fluorescent activity.
Figure 17:
Table 1: Primers used for Q-PCR analysis:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon</th>
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<tr>
<td>Tom40 (NM_001109748)</td>
<td>CGGTGTGGATGGCGGAGTACG</td>
<td>CCTGAAACCACCCAAAGCATCTG</td>
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<tr>
<td>Tom22 (NM_172609)</td>
<td>CGGGCCGAGGAAATTACGCTCCCAGC</td>
<td>CGTCTTCTTCCAGCTCCTCCTC</td>
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<tr>
<td>Tim13 (NM_013898)</td>
<td>CGATAGGCTCCTTGGAATACTCG</td>
<td>AGAGTTGTAGGCGCGGGACAC</td>
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<tr>
<td>Tim22 (NM_019818)</td>
<td>GTACCTGGTGAGGCGACAGAC</td>
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<tr>
<td>Sam50 (NM_178614)</td>
<td>GTGCATCCGCTTGCTCCTGAT</td>
<td>CGCGGTAGTGCCAGCTCCAGCG</td>
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<td>Metaxin 1 (NM_013604)</td>
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<td>Metaxin 2 (NM_016804)</td>
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<td>HPRT (NM_013556)</td>
<td>GTACCAGACCTCTCGAAGTGGGGATA</td>
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Table 2: Identification of proteins present in the 66- and 800 kDa mitochondrial protein complexes by mass spectrometry in HeLa cells. The 66- and 800 kDa mitochondrial protein complexes separated by BN-PAGE and 2D SDS-PAGE were analyzed by mass spectrometry; some of the major identified proteins are shown.
Table 2:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequences Identified</th>
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<tbody>
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<td><strong>66 kDa</strong></td>
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<tr>
<td>Metaxin1</td>
<td>RSLASPGISPGPLTATIGGAVAGGGPR</td>
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<tr>
<td>Nonspecific lipid-transfer protein</td>
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<td></td>
<td>K.IGGIFAFK.V</td>
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<tr>
<td></td>
<td>K.ANLVFKEIEK.K</td>
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<tr>
<td>Voltage-dependent anion channel (VDAC 1)</td>
<td>TDEFQLHTNVNDGTEFGGSIYQK</td>
</tr>
<tr>
<td></td>
<td>R.WTEYGLTFTEK.W</td>
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<tr>
<td></td>
<td>K.LTFDSSFSPTNGK.K</td>
</tr>
<tr>
<td><strong>800 kDa</strong></td>
<td></td>
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<tr>
<td>Adenine nucleotide translocator (ANT 1,2,3)</td>
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<td></td>
<td>R.YFPTQALNFAFK.D</td>
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<td>R.AAYFGIYDTAK.G</td>
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<td>K.DFLAGGIAAAISK.T</td>
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<td>Apolipoprotein A-II</td>
<td>K.SPELQAEAK.S</td>
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<tr>
<td>Fatty acid synthase</td>
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<td>K.VGDPQELENGITR.A</td>
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<td>Mitofilin</td>
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<td>Voltage-dependent anion channel (VDAC 1,3)</td>
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<td></td>
<td>K.LSQNNFALGYK.A</td>
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<td><strong>66 and 800 kDa</strong></td>
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<td></td>
<td>K.GVDEVTIVNILTNR.S</td>
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<td>Phosphatidylinositol 4,5-biphosphate-dependent ARF1</td>
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<tr>
<td>GTPase-activating protein</td>
<td>R.QEEIDESDDDLDDK.P</td>
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<td></td>
<td>K.NGILTISHTSNR.Q</td>
</tr>
<tr>
<td></td>
<td>K.SFDLISHNR.T</td>
</tr>
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</table>
Table 3: Identification of proteins present in the 66 and 800 kDa mitochondrial protein complexes in control and treated MA-10 cells by mass spectrometry. The 66- and 800 kDa mitochondrial protein complexes separated by BN-PAGE and 2D SDS-PAGE were analyzed by mass spectrometry; some of the major identified proteins are shown.
Table 3:

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<th>Protein:</th>
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<tr>
<td><em>Ras-related proteins:</em> Rab 1A, Rab 5C, Rab 7, Rab 14</td>
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<tr>
<td><em>14-3-3 Proteins:</em> Epsilon, Gamma</td>
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<td><em>Steroidogenic Proteins:</em> CYP11A1, Acyl-CoA-binding protein 1 (ACBP1)</td>
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<td><em>Mitochondrial import proteins:</em> Tim 8, Tim 13, Tim 23</td>
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<td><em>Various proteins:</em> Oxysterol-binding protein-related protein 1</td>
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<td><strong>140 kDa</strong></td>
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<td><em>ER-Mitochondria Contact Site:</em> Inositol 1,4,5-trisphosphate receptor, VDAC1, 78 kDa glucose-regulated protein precursor (GRP 78)</td>
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<td><em>Golgi-associated retrograde protein (GARP) complex:</em> VPS52, VPS53</td>
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<td><em>Various proteins:</em> Apolipoprotein A-IV, Calnexin Heat shock 70 kDa protein, Superoxide dismutase</td>
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Reference List


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