MITOCHONDRIAL PROTEINS AT EXTRAMITOCHONDRIAL LOCATIONS

IMMUNOELECTRON MICROSCOPY PROVIDES EVIDENCE FOR THE EXTRAMITOCHONDRIAL LOCALIZATION OF A NUMBER OF NUCLEAR- AND MITOCHONDRIAL- DNA ENCODED PROTEINS

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ABSTRACT

Mitochondrial proteins are presently believed to reside and function only within the mitochondria, under normal physiological conditions. However, in recent years many examples have come to light where proteins originally identified on the basis of extramitochondrial functions, upon characterization were found to be bonafide mitochondrial proteins. This raises important questions concerning the cellular functions of mitochondrial proteins. To investigate this phenomenon of mitochondrial proteins being present at extramitochondrial locations, we have examined by means of immunoelectron microscopy, the subcellular localization in normal rat tissues of a number of well characterized mitochondrial proteins which are encoded either by the nucleus (chaperonin 10 (Cpn10) and mitochondrial aspartate aminotransferase (mAspAT)), or by mitochondrial DNA (Cytochrome c oxidase subunits I and II (COX I and II)), using highly specific antibodies. Both Cpn10 and mAspAT have already been shown to be involved in extramitochondrial functions. Cpn10 has been shown to be identical to early pregnancy factor, which is an immunosuppressant and growth factor found in maternal serum. mAspAT is identical to fatty acid binding protein isolated from plasma membranes of several cell types, involved in the transport of long chain free fatty acids.

Immunofluorescent labeling of BS-C-1 African monkey kidney cells with these antibodies showed characteristic mitochondrial labeling. In all tissues examined, the antibodies showed strong labeling of mitochondria, as was expected. In several tissues,

the binding was seen exclusively within mitochondria. However, in a variety of tissues such as pancreas, anterior pituitary and kidney, these antibodies showed strong and specific labeling of one or more of the following compartments- pancreatic zymogen granules, growth hormone granules, secretory granules in islet cells, red blood cells, condensing vacuoles in kidney and on the cell surface of different regions of the kidney. All of the observed labeling with these antibodies, both within mitochondria and in other compartments, was abolished upon omitting the primary antibodies or upon adsorption of the Cpn10 antibody (in the case of Cpn10) with purified recombinant protein. The extramitochondrial localization of these proteins, particularly those encoded by mitochondrial DNA provides strong evidence that these proteins have reached these sites by exiting from mitochondria. These observations have important implications concerning the roles of mitochondria and mitochondrial resident proteins in different mitochondrial diseases. Also, the function of these proteins and the precise mechanism by which they reach these extramitochondrial sites is of great interest.

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LIST OF ABBREVIATIONS

μg	microgram
μm	micrometer
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
Apaf-1	apoptotic protease activating factor 1
ATP	adenosine triphosphate
BSA	bovine serum albumin
CHO	Chinese hamster ovary
COX	cytochrome c oxidase
Cpn 10	chaperonin 10
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EPF	early pregnancy factor
ER	endoplasmic reticulum
FFA	long chain free fatty acid
$FABP_{PM}$	plasma membrane fatty acid binding protein
FCS	fetal calf serum
g	gram
GTP	guanosine triphosphate
Hsp	heat shock protein
kDa	kilodalton
M	molar
mAspAT	mitochondrial aspartate aminotransferase
MELAS	mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes
MEM	minimal essential medium
mg	milligram
mHsp70	mitochondrial heat shock protein 70
ml	milliliter
mm	millimeter
mM	millimolar
MPT	mitochondrial permeability transition
MTF	mitochondrially transmitted factor
NADH	nicotinamide adenine dinucleotide
NARP	neuropathy ataxia and retinitis pigmentosa
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBP	peptide binding protein
RNA	ribonucleic acid

ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
s	seconds
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TBS	tris buffered saline
TIM	translocase of the innermembrane
TOM	translocase of the outermembrane
TRAP-1	tumor necrosis factor receptor associated protein 1
tRNA	transfer ribonucleic acid
VDAC	voltage dependent anion channel
vol	volume
wt	weight
ZG	zymogen granule

Chapter 1.0 :

General Introduction

1.1 Current View of Mitochondria

Mitochondria are present virtually in all eukaryotic species. It is generally accepted that mitochondria evolved from gram negative proteobacteria via endosymbiotic event nearly 1.5 billion years ago, whereby it was engulfed by a protoeukaryotic cell (Margulis, 1970; Gray and Doolittle, 1982; Gray, 1992; Margulis, 1993). The many features of mitochondria that reflect its endosymbiotic origin are: (i) Double membrane consisting of an outer and inner membrane, (ii) Circular mitochondrial genome and (iii) Mitochondria specific transcription and translation system. Over the years the mitochondrial genome size has been reduced with the transfer of essential genes to the nucleus. The nucleus thus encodes for a variety of mitochondrial proteins including proteins involved in oxidative phosphorylation, DNA and RNA polymerases, ribosomal proteins and mitochondrial DNA regulatory factors. Thus, these proteins have to be transcribed in the nucleus, translated in the cytosol and then imported into either the mitochondrial outermembrane, innermembrane, matrix or intermembrane space (Schatz, 1996). The current assumption is that once these proteins have been imported into the mitochondria they reside and function within the mitochondria under normal physiological conditions. Knowing that mitochondria are comprised of two membranes has led to the belief that these proteins cannot have external functions.

The human mitochondrial DNA is 16, 569 bp in length and encodes for a total of 37 genes, out of which 13 encode for proteins all of which are present in mitochondrial inner membrane and are involved in oxidative phosphorylation machinery (Anderson et al., 1981). These proteins include 7 subunits of NADH dehydrogenase complex, 3

subunits of the cytochrome c oxidase complex, 2 subunits of ATP synthase F_0 sector and cytochrome b. It also encodes the 12S and 16S rRNA genes and 22 tRNA genes involved in protein translation within the mitochondria (Gray et al., 1999). One of the functions carried out by the mitochondria is to harness ATP (~30 ATP molecules / glucose molecule). This is achieved by the chemiosmotic theory proposed by Peter Mitchell whereby the transfer of electrons along the electron transport chain is coupled to the extrusion of protons into the intermembrane space (Mitchell, 1979). As a result, there is a pH gradient build up, with the matrix being alkaline and the intermembrane space being acidic. Also, due to the gradient, there is a membrane electrical potential whereby the inside is negative and the intermembrane space is positive. Both the pH gradient and the membrane electrical potential contribute to the proton motive force which is used to catalyze the synthesis of ATP (Lehninger et al., 1993). Complex I or NADH dehydrogenase complex is comprised of atleast 42 subunits, with 35 being encoded by the nucleus. It is the largest complex in the electron transport chain and is involved in reducing NADH and transferring the electrons to ubiquinone or coenzyme Q_{10}, while pumping protons into the intermembrane space (Lehninger et al., 1993). Complex II or Succinate dehydrogenase is made up of 4 subunits all of which are encoded by the nucleus. It is involved in converting succinate to fumarate while transferring electrons to ubiquinone. This is the only enzyme in the citric acid cycle that is membrane bound and thus links the citric acid cycle with the electron transport chain (Lehninger et al., 1993). Complex III or ubiquinone-cytochrome c oxidoreductase is involved in the transfer of electrons from ubiquinone to cytochrome c with pumping of

protons into the intermembrane space. Complex III is comprised of a total of 11 subunits, of which 10 are encoded by the nucleus (Lehninger et al., 1993). Complex IV or cytochrome c oxidase is involved in the transfer of electrons from cytochrome c to oxygen making water (Michel et al., 1998). The transfer of electrons is accompanied by the pumping of protons from the matrix to the intermembrane space. Complex IV is composed of 13 subunits of which 10 are encoded by the nucleus. Complex V or ATP synthase is made of at least 16 subunits of which about 14 are encoded by the nucleus. It is made up of two segments, the F_1 hydrophilic catalytic segment and the F_0 hydrophobic proton translocating segment. It is involved in using the proton motive force to catalyze the synthesis of ATP. with the flow of protons from the intermembrane space into the matrix (Stock et al., 1999).

Mitochondria have sometimes been described in some biochemistry textbooks as 'cigar' or 'sausage' shaped organelles with a diameter of 1 micrometer (μm). On the contrary, mitochondria are very dynamic structures undergoing constant fission and fusion, forming extended reticular networks (Yaffe, 1999). The distribution and morphology of mitochondria in different tissues, is highly variable and is also brought about by key stages in cell cycle progression and differentiation. Some examples illustrating the above mentioned points are as follows: (i) Most of the cells in our body contain anywhere from 500-2000 mitochondria on an average (Lehninger et al., 1993). Cone cell photoreceptors of the eye are made up of mostly mitochondria, whereas platelets have 2-6 and red blood cells have none (Weiss, 1988). (ii) In heart muscle and kidney tubules mitochondria are localized to specific regions of the cytoplasm whereas in

sperm tail, mitochondria are wrapped around the axoneme in a helical conformation (Capaldi, 2000). (iii) Osteosarcoma cells that have been transfected with green fluorescent protein targeted to the matrix show fragmented mitochondria during the S phase which form extended reticular networks during the G1 phase of the cell cycle (Capaldi, 2000). Cytoskeleton play an important role in the movement of mitochondria as mitochondria co-localize with the cytoskeletal elements, especially microtubules in a variety of cell types (Heggeness et al., 1978; Yaffe et al., 1996). Evidence for this also comes from the finding that mitochondria rearrange when cells are treated with anti-mitotic drugs that disrupt microtubules (Ball and Singer, 1982).

Mitochondria undergo fission and fusion which could result in exchange or the mixing up of proteins when such processes occur. 'Fuzzy Onions' (fzo) gene encodes a GTPase that spans the outermembrane with its GTPase activity in the cytoplasmic domain and is found in a variety of mammalian tissues and thought to aid in mitochondrial fusion (Rapaport et al., 1998). In yeast, mutation in Fzo1p gene leads to fragmentation of mitochondria and loss of mitochondiral DNA (Hermann et al., 1998). The exact mechanism by which fusion occurs is not known, Fzo1p could be a part of a multi protein fusion complex or it may be involved in initial membrane contact. The fusion process is probably complex as it requires the joining of both the inner and outer membranes (Rapaport et al., 1998; Thomson, 1998). In yeast several Mdm (mitochondrial distribution and morphology) proteins have also been shown to be present in the outermembrane of mitochondria and are responsible for the interaction of mitochondria with the cytoskeletal elements (Yaffe, 1999; Berger and Yaffe, 1996).

Prior to the 1980's the sole function of mitochondria has been thought to be to harness energy, hence they have been referred to as 'energy centers' or 'power house of the cell'. Out of the many genes necessary to put together a mitochondria only a small fraction of genes are involved in the production of ATP. The remaining genes are involved in numerous specific functions in differentiated cells (Wallace, 1999). Mitochondria are involved in numerous functions such as pyrimidine biosynthesis essential for RNA and DNA, heme synthesis, break down of ammonia in urea cycle, cholesterol metabolism, key role in regulation of calcium levels and signaling in the cell, and involved in the biosynthesis of [Fe-S] proteins located in the mitochondria, nucleus and cytosol (Lehninger et al., 1993). The interest in this organelle has grown substantially over the past twenty years with two main developments: (i) Characterization of the genetic basis of a large number of mitochondrial diseases (Schon, 2000) and (ii) Mitochondria have been shown to be a central player in apoptosis or programmed cell death (Susin et al., 1998). Thus, the structural and functional aspects of this organelle are undergoing major changes in recent years and hence in an editorial in *TiBS* it was commented, "mitochondrial structure and function is leading to a redefining of long-held views about the basic biology of the organelle (Capaldi, 2000)."

1.2 Protein Import into the Mitochondria

Mitochondrial proteins encoded by the nuclear DNA are translated on cytosolic ribosomes and then translocated either into the mitochondrial matrix, innermembrane, intermembrane space or outermembrane, based on their targeting sequence (Neupert,

1997). During and after translation on ribosomes cytosolic Hsp70 bind to the preproteins, preventing aggregation and keeping them translocation competent; (Chirico et al., 1988; Murakami et al., 1988) thus, aiding in either co- or post-translational import. However, it has been suggested that cotranslational import would be an extremely slow process (Neupert, 1997). Most mitochondrial proteins are synthesized with a N-terminal targeting sequence which is cleaved upon import into the mitochondria by a matrix resident signal peptidase (Hendrick et al., 1989). There is no established consensus sequence for mitochondrial targeting, however they have positively charged amino acid residues, have the ability to form an amphipathic helix (Hammen and Weiner, 1998) and are about 20-60 amino acids in length. Protein translocation to the matrix, innermembrane and intermembrane space but not outermembrane space requires membrane potential. However there are many exceptions to the above mentioned rules and some examples are: (i) Hsp10/Cpn10 has a matrix targeting sequence, but is not cleaved upon import (Ryan et al., 1995), (ii) Proteins of the outer and inner mitochondrial membrane generally have internal targeting sequence (Neupert, 1997), (iii) Yeast mitochondrial matrix protein MTF1 does not have a characteristic N-terminal presequence and its import is not dependent on the outermembrane translocase complex, membrane potential or ATP (Sanyal and Getz, 1995).

Translocase of the outermembrane (TOM) and translocase of the innermembrane (TIM) are the translocases of the mitochondria that aid in the import of proteins. TOM receptors which are TOM 20, 22, 37 and 70 recognize the mitochondrial targeting sequence and TOM 40, 5, 6 and 7 form the import channel for the passage of

the protein (Lithgow, 2000). Once the protein has passed through the TOM complex it can either be: (i) Inserted into the outermembrane, (ii) Directly released into the intermembrane space, (iii) Passed on to the TIM23 complex which translocates the protein into the matrix or (iv) Passed on to the TIM22 complex which aids in the insertion of the protein into the innermembrane space (Koehler, 2000).

The positively charged N-terminus of proteins destined to the matrix bind to the negatively charged domain of TIM23 (which is a part of TIM23/TIM17), leading to the transient interaction of TIM23/TIM17 and TOM complexes (Koehler, 2000). This forms a channel for the protein to be translocated from the cytoplasm to the mitochondrial matrix without release into the intermembrane space (Berthold et al., 1995). The translocation motor apparatus is comprised of mitochondrial (m)-Hsp70, mGrpE and TIM44. About 10% of mHsp70 in the matrix is bound to TIM44, which in turn is associated with the innermembrane on the matrix side (Neupert, 1997). mGrpE acts as a nucleotide exchange factor aiding in the release of nucleotides bound to mHsp70 (Schneider et al., 1996). This translocation motor ensures vectorial import of proteins into the mitochondrial matrix. There are three models that have been proposed to describe the action of mHsp70: (i) Brownian ratchet (Ungermann et al., 1996; Gaume et al., 1998): This model proposes that the preprotein in the TIM and TOM complexes is free to diffuse in and out. Binding of mHsp70 to the proprotein every time it moves into the matrix ensures vectorial transport into the mitochondria. (ii) Translocation motor (Horst et al., 1996): In this model the conformational change brought about in mHsp70 by the hydrolysis of ATP is coupled to the 'pulling' the preprotein into the matrix. (iii)

This model proposes that the action of mHsp70 could be a combination of both the Brownian ratchet and Translocation motor models (Voisine et al., 1999).

The proteins that are destined to the innermembrane space are passed from the TOM to TIM22 complex comprised of TIM [10, 9, 12, 18, 22, 54], which aids in the insertion into the innermembrane (Koehler, 2000; Leuenberger et al., 1999). Since these hydrophobic proteins have to cross the hydrophilic intermembrane space, TIM 9/10 and TIM 8/13 which are present in the intermembrane space provide chaperone like function aiding in the translocation of innermembrane proteins through the intermembrane space (Koehler, 2000). It is interesting to note that loss of TIM 8 function leads to a X-linked neurodegenerative disease called Mohr-Tranebjaerg syndrome, with symptoms like deafness, dystonia, dementia and blindness (Tranebjaerg et al., 1995; Jin et al., 1996). This stresses the importance of proper import and sorting of mitochondrial proteins.

Proteins encoded by the mitochondria are transcribed and translated on mitochondrial ribosomes and exported to the innermembrane by Oxa1 (Hell et al., 1997; Hell et al., 1998) and Pnt1 (He and Fox, 1999) both of which are present in the innermembrane. Oxa1 and Pnt1 are both involved in the export of mitochondrially encoded cytochrome c oxidase subunit II (COX II) from the matrix to the innermembrane.

1.3 Role of Mitochondria in Apoptosis

There are a variety of agents that can induce programmed cell death or apoptosis such as depletion of growth factors, rise in calcium concentrations, ionizing irradiation,

heat shock and drugs such as etoposide, actinomycin D, staurosporine and anti-mitotic drugs.

Mitochondria are a key player in apoptosis in that it is involved in the activation of caspases by the release of cytochrome c which is present in the intermembrane space to the cytosol (Green and Reed, 1998; Desagher and Martinou, 2000; Brenner and Kroemer, 2000). Caspases are a family of cysteine proteases that cleave specifically after aspartic acid residue. They are synthesized as proenzymes and upon proteolytic cleavage become active and cleave other substrates within the cell (Thornberry and Lazebnik, 1998).

The released cytochrome c in the cytosol forms a complex with apoptotic protease activating factor 1 (Apaf-1) and procaspase-9, called the apoptosome. In the presence of ATP procaspase-9 is converted to active caspase-9 which in turn is involved in the clevage and activation of procaspase-3. The series of biochemical events that follow lead to characteristic apoptosis which include nuclear shrinkage, DNA fragmentation, membrane blebbing and the appearance of apoptotic bodies (Green and Reed, 1998; Desagher and Martinou, 2000; Brenner and Kroemer, 2000). It is interesting to note that mitochondria also releases apoptosis inducing factor (AIF) which is an intermembrane space protein into the cytosol in response to apoptotic stimuli. AIF in turn is translocated to the nucleus and mediates apoptosis in a caspase independent pathway (Daugas et al., 2000a).

The Bcl-2 family of proteins play an important role in the release of proteins from mitochondria (Adams and Cory, 1998). The Bcl-2 family of proteins are divided

into two groups one that is pro-apoptotic and the other is anti-apoptotic, which can homo- or hetero-oligomerize with each other. The pro-apoptotic members include Bax, Bid, Bim and anti-apoptotic members include Bcl-2 and Bcl-x_L. The control for apoptosis depends on the phosphorylation states of these proteins and the relative concentrations of these proteins (Green and Reed, 1998; Desagher and Martinou, 2000; Brenner and Kroemer, 2000). Bcl-2 is membrane associated (cytoplasmic side) via the C-terminal hydrophobic domain with the mitochondrial outermembrane, endoplasmic reticulum and nuclear envelope (Adams and Cory, 1998). Whereas members of the proapoptotic family Bax, Bid and Bim translocate from the cytosol to mitochondria during apoptosis (Porter, 1999).

Several models have been proposed to explain the release of cytochrome c into the cytosol. The first model is where, during apoptosis Bax translocates from the cytosol to the mitochondrial outermembrane, oligomerizes and either alone or in conjunction with voltage dependent anion channel (VDAC)/porin forms channels in the outermembrane enabling the release of cytochrome c (Green and Reed, 1998; Desagher and Martinou, 2000; Porter, 1999). This model is based on the finding that Bcl-2 and Bid are able to form channels for ions and proteins in synthetic membranes (Schendel et al., 1998; Antonsson et al., 2000).

The second model describes a process by which there is non-specific release of intermembrane space proteins due to the swelling of the matrix and eventual rupture of the outermembrane (Green and Reed, 1998; Desagher and Martinou, 2000). The innermembrane does not rupture because it is highly folded into structures called cristae

and thus provides a large surface area to accommodate the swelling. One possibility is the rupture of the outermembrane due to the closure of VDAC and impairment of the functioning of adenine nucleotide translocator (ANT), which is involved in the ATP/ADP exchange (Vander Heiden et al., 1999; Desagher and Martinou, 2000). As a result, there would be a build up of protons in the intermembrane space resulting in the hyperpolarization of the innermembrane thus leading to the swelling of the matrix and the eventual rupture of the outermembrane. The second possibility is the release of mitochondrial proteins due to mitochondrial permeability transition (MPT) (Crompton, 1999; Desagher and Martinou, 2000), which increases the permeablility of innermembrane, allowing the free passage of molecules less than 1500 Da. This leads to loss of membrane potential and causes matrix swelling, followed by rupture of outermembrane and the release of intermembrane space proteins into the cytosol. It has been suggested that MPT involves an interaction of innermembrane ANT, outermembrane VDAC/porin, cytosolic hexokinase and mitochondrial creatine kinase (Desagher and Martinou, 2000). Calcium, Bax and reactive oxygen species (ROS) are known to favour the opening of the MPT (Crompton, 1999).

Interestingly, Hsp60 and its co-factor Hsp10/Cpn10 have been shown to be associated with procaspase-3 in the intermembrane space of mitochondria and upon the induction of apoptosis have been shown to be involved in their activation (Xanthoudakis et al., 1999; Samali et al., 1999). Unfortunately, none of the models discussed above can account for the release of matrix proteins into the cytosol.

1.4 Mitochondrial Diseases

Mitochondria have been implicated in a broad spectrum of genetic diseases such as myopathies, neuropathies, encephalomyopathies, cardiomyopathy, deafness, Alzheimer's diseases, Parkinson's disease, Huntington's disease, diabetes mellitus and aging (as a general process) (Larsson and Luft, 1999; Wallace, 1999).

Mitochondrial mutations can be classified into two general classes, the first class consists of diseases arising from major deletions of one or more genes encoding for either electron transport chain proteins, tRNA or rRNA (Schon, 2000). The diseases that arise from this class of mutation can withstand only low levels of heteroplasmy. A typical cell has on an average 100 mitochondria and each mitochondria may contain 2-10 molecules of DNA. Thus, heteroplasmy is defined as the percentage of mutated mitochondrial DNA to wild type mitochondrial DNA in a cell or tissue. Mitochondrial DNA deletions could occur for genes encoding proteins that function in all of the complexes of the electron transport chain except Complex II, as all the subunits for this complex is encoded for by the nuclear DNA. Some examples of diseases associated with large mitochondrial deletions are Kearns-Sayre syndrome and Progressive external ophthalmoplegia (Holt et al., 1988). The second class of diseases arises from specific point mutations in the mitochondrial genome that might occur in a gene encoding for electron transport chain proteins, tRNA or rRNA (Schon, 2000). These mutations are sometimes more forgiving than large deletions and patients often have high level of heteroplasmy without clinical symptoms. The reason being, the mutation might be such

that the function of the protein or tRNA is not totally disabled giving rise to residual wild type activity.

A unique feature of mitochondrial diseases is that identical mitochondrial mutations may not present with same disease phenotype and different mutations in mitochondrial DNA could often give rise to same disease phenotype. This makes diagnosis of mitochondrial diseases challenging and therefore it is easy to classify the disease by genetic defect rather than phenotypic traits (Wallace, 1999). Examples to illustrate the above points are as follows: (i) The point mutation T8993G/C in mitochondrial encoded ATP synthase subunit 6 leads to two different disease phenotypes depending on the level of heteroplasmy (Holt et al., 1990). Lower levels of heteroplasmy results in NARP, which shows symptoms like neuropathy, ataxia, retinitis pigmentosa, dementia and developmental defects. Higher levels of heteroplasmy gives rise to Leigh syndrome like symptoms which include brain dysfunction and elevated lactic acid levels in blood (Shoffner et al., 1992; Ortiz et al., 1993). (ii) MELAS which presents with symptoms like lactic acidosis, stroke like episodes, seizures, mental retardation and hearing loss. MELAS can be caused either by a point mutation in $tRNA^{Leu}$ gene (A3243G) or by a mutation in the cytochrome c oxidase subunit III gene (Goto et al., 1990; Ortiz et al., 1993). Usually the displayed clinical phenotype is determined by the level of heteroplasmy at any given time and the distribution of the heteroplasmy in different tissues in the body. However it is important to note that the level of phenotypic presentation of the disease is not related to the mutational load in a linear fashion (Wallace, 1999; Schon, 2000).

In mitochondrial diseases, different tissues can withstand different levels of heteroplasmy because each tissue has a unique oxidative phosphorylation threshold for its optimal functioning and this is called the 'threshold effect' (Rossignol et al., 1999). Thus, organs that have high energy requirements are the most sensitive to even small proportions of heteroplasmy and these include brain, heart, skeletal muscle, kidney and insulin secreting pancreatic β-cell, which are often affected in mitochondrial diseases (Fosslien, 2001).

Unlike nuclear DNA where there are only two copies per gene, mitochondrial genes are present in thousands of copies in a typical cell, all of which are maternally inherited. The sperm does contribute nearly hundred mitochondria for every fertilized egg, however the mitochondria have ubiquitin tag for degradation by the embryo's proteasomes and lysosomes (Sutovsky et al., 1999). The fertilized egg contains approximately hundred thousand mitochondrial DNA molecules, of these only thousand will segregate randomly into the fetus's tissues. Thus, the number of mutated mitochondria segregated to each tissue in embryogenesis is a random event and it is unlikely that there is any selection pressure (Jenuth et al., 1996; Wallace, 1999; Schon, 2000). This explains the varied levels of heteroplasmy in different tissues observed in patients with mitochondrial diseases.

Oxidative phosphorylation is a major source of reactive oxygen species (ROS), such as H_2O_2, $OH^{\cdot}$, $O_2^{\cdot-}$ (Chance et al., 1979; Papa and Skulachev, 1997), especially when there is a defect in the electron transport chain Complex I (Robinson, 1998). Cells are equipped with superoxide dismutases (SOD) to neutralize the ROS. In the cytosol there

are Cu, Zn-SOD and in the mitochondria there is Mn-SOD. In mitochondria ROS are neutralized by Mn-SOD which converts it to H_2O_2 and glutathione peroxidase converts H_2O_2 to water (Gutteridge, 1994). ROS cause damage to proteins, DNA, lipids and [Fe-S] clusters (Fosslien, 2001). Thus, mitochondria have a higher propensity to accumulate mutations/damage because of : (i) Close proximity of the mitochondrial DNA to the electron transport chain, (ii) Unlike nuclear DNA, mitochondrial DNA does not have histones associated with it, (iii) Mitochondria is known to have an inefficient DNA repair system (Sawyer and Van Houten, 1999; Wallace, 1999; Fosslien, 2001). As a result the mitochondrial DNA has a ten times higher mutational rate than nuclear DNA (Brown et al., 1979; Brown et al., 1982). Thus, accumulation of mitochondrial DNA mutations during ones life span is implied in the general process of aging (Linnane et al., 1989; Beckman and Ames, 1998). Mitochondrial DNA from cortical brain region of patients with Huntington's and Alzheimer's disease show increased mitochondrial deletions (Corral-Debrinski et al., 1994; Horton et al., 1995), with the latter also showing oxidative damage (Mecocci et al., 1994).

Even though there are number of models to explain different aspects of mitochondrial diseases, it is still puzzling to explain how a mutation or deletion of a mitochondrial gene involved in oxidative phosphorylation could give rise to such wide ranging clinical phenotypes, tissue specificity and varied onset of the disease.

1.5 Proteins with Multiple Functions

The notion of one gene, one protein, one function is too simplistic to explain cellular processes (Smalheiser, 1996; Jeffery, 1999; Soltys and Gupta, 1999). A large number of proteins have been identified to be involved in two or more functions. These proteins are encoded by a single gene and for all of them no closely related homologs are found. There is also no evidence for alternate transcription or, alternate mRNA translation or splicing. These proteins are involved in different functions due to either change in cellular localization (both in and out of the cell), oligomerization/complex formation or the cell type (Jeffery, 1999). This is not restricted to one class of proteins but to a wide variety of proteins involved in different functions. Some examples are growth factors, cytoskeletal proteins, glycolytic proteins and chaperones (Smalheiser, 1996). As it was stated by Smalheiser (Smalheiser, 1996), "Multicompartmentalization of proteins appears to be a real phenomenon and is not explainable, in general, by artificats such as movement of proteins during isolation, contamination by proteins released from dead/wounded cells, or adventitious cross-reactions of antibodies with unrelated proteins."

Some examples of proteins with multiple functions are as follows. Phosphoglucose isomerase which plays an important role in glycolysis, is also present outside the cell where it functions as a nerve growth factor (Chaput et al., 1988; Faik et al., 1988). TIM11 which is a part of the translocase machinery present in the mitochondrial innermembrane has been shown to be identical to nuclear encoded ATP synthase subunit γ, which functions as an assembly factor for ATP synthase (Arnold et al., 1998). Nuclear encoded ATP synthase subunit α has been shown to be induced under

heat shock conditions in larvae of *Drosophila hydei* (Luis et al., 1990). It also exhibits high identity to some of the conserved amino acids in molecular chaperones and is thought to also function as a chaperone within the mitochondria (Luis et al., 1990). Interestingly, ATP synthase subunit α has also shown to be present in rat liver peroxisomes, its function in this compartment remains to be elucidated (Cuezva et al., 1990). Thus, ATP synthase subunits γ and α which are thought to be primarily involved in the mitochondrial oxidative phosphorylation, also have other additional functions both within and outside mitochondria.

1.6 Mitochondrial Proteins at Extramitochondrial Sites

A general belief is that once proteins are imported into mitochondria they reside and function within the organelle under normal physiological conditions. This belief is in large part due to the fact that mitochondria are bounded by two membranes and no mechanisms are presently known for protein export across these membranes. The localization of a given protein to mitochondria is generally based on criteria such as immunofluorescence microscopy, presence of a mitochondrial targeting sequence and cell fractionation studies. Using these techniques, the presence of small amounts of mitochondrial proteins at extramitochondrial sites cannot be resolved. Recently, a large number of proteins that have been identified based on their extramitochondrial function, have turned out to be mitochondrial proteins upon characterization. Our lab and others have shown numerous mitochondrial proteins to be present at specific extramitochondrial locations under normal cellular conditions. These proteins are encoded by a single

nuclear gene and there is no evidence for alternate transcription or alternate mRNA translation or splicing (Danpure, 1995).

Some examples of nuclear encoded mitochondrial proteins found at extramitochondrial locations are described below:

Mitochondrial Matrix Proteins

Mitochondrial Heat Shock Proteins

Three well studied mitochondrial matrix heat shock proteins are Hsp60, mHsp70 and Hsp10/Cpn10 (co-factor for Hsp60). These proteins are synthesized with a N-terminal targeting sequence, which is cleaved upon import into the mitochondrial matrix (except for Cpn10) (Ryan et al., 1995). Even though they are called heat shock proteins due to their thermal inducibility, they are essential under all cellular conditions (Parsell et al., 1993; Georgopoulos and Welch, 1993; Ellis and van der Vies, 1991). Hsp60 and mHsp70 are involved in the correct folding of proteins into monomeric or oligomeric complexes, thus preventing aggregation or misfolding (Ellis and van der Vies, 1991; Craig et al., 1993; Hartl, 1996). Mitochondrial Hsp70 is also involved in the import of proteins across the outer and inner mitochondrial membranes in a partially unfolded state (Ellis and Hartl, 1996; Neupert, 1997).

Hsp60

Evidence for the presence of Hsp60 on cell surface of different cell types comes from the observation that stressed macrophages and certain tumor cells that are blocked with specific antibodies to Hsp60 are no longer able to stimulate T cells (Kaur et al., 1993). Also, biotinylation (Soltys and Gupta, 1996) and radio-iodination (Kaur et al.,

1993; Fisch et al., 1990) of cell surface proteins in Chinese hamster ovary (CHO) cells and Daudi lymphoma cells respectively, have shown the presence of Hsp60 on cell surface.

In our lab, Hsp60 has been implied in conferring resistance to mutant cells that are resistant to anti-mitotic drugs such as podophyllotoxin, which inhibit mitotic spindle formation and function. CHO cell mutants that are resistant to podophyllotoxin show altered mobility of Hsp60 on 2-D gels (Gupta et al., 1982; Gupta et al., 1985). Hsp60 has been shown to be co-released with tubulin under conditions which depolymerize microtubules. Furthermore, the mutant cell extracts show reduced binding to ^{3}H-podophyllotoxin compared to wild type suggesting that the mutation either directly or indirectly affects drug binding to tubulin. The altered mobility of Hsp60 has been shown to be due to a single base change which leads to a Gly to Glu change at position 33 (Gupta et al., 1991). Presence of Hsp60 and tubulin on cell surface might explain how a mitochondrial matrix protein could lead to the resistance to a drug that binds to tubulin. It is suggested that tubulin on the cell surface provides the main target for the antimitotic drugs. Interaction of mutant Hsp60 with tubulin prevents drug binding to tubulin leading to drug resistance (Estridge, 1977; Rubin et al., 1982; Quillen et al., 1985; Stephen, 1986).

Immunoelectron microscopic studies performed on rat tissues have shown Hsp60 to be present in both mitochondria and at extramitochondrial locations such as the plasma membrane, endoplasmic reticulum, peroxisomes, cytosolic vesicles, mature insulin secretory granules, zymogen granules and growth hormone granules (Soltys and

Gupta, 1996; Cechetto et al., 2000; Brudzynski et al., 1992). It has been hypothesized that the function of Hsp60 in these granules is to act as a molecular chaperone (Soltys and Gupta, 2000). For example, zymogen granules that are found in the pancreatic acinar cells, contain a variety of inactive precursor digestive enzymes such as amylase, chymotrypsinogen and trypsinogen. It has been suggested that Hsp60 functions in packaging and preventing the premature activation of these precursor proteins (Le Gall and Bendayan, 1996; Cechetto et al., 2000). Presence of Hsp60 in purified zymogen granules has also been confirmed by western blotting and immunoprecipitation (Le Gall and Bendayan, 1996; Cechetto et al., 2000). It should be noted that a number of specific monoclonal and polyclonal antibodies were used in these studies. The specificity of these antibodies were analyzed using a number of methods including 1- and 2-D gel blots (Gupta and Dudani, 1987), immunoprecipitation and preadsorption of the antibodies with purified recombinant Hsp60 completely abolished labeling in immunoelectron microscopy. Furthermore, these antibodies show no cross reactivity with TCP-1 family of proteins which function as chaperones in the assembly of cytoskeletal proteins and are distantly related (≈20% amino acid identity) to Hsp60 (Gupta, 1995).

The involvement of Hsp60 in cell surface associated functions has also been indicated by a variety of other studies. Khan *et al.* (1998), have found histone 2B to be present as a complex with Hsp60 in the plasma membrane of T cells. Phosphorylation by type 1 protein kinase A of both Hsp60 and histone 2B leads to the disruption of the complex and release of only histone 2B from the plasma membrane. Hsp60 in this case is thought to be acting as a chaperone to facilitate membrane association of a soluble

histone 2B protein. The function of histone 2B (a nuclear protein) at this location is not known (Khan et al., 1998). These studies also provide evidence that Hsp60 on cell surface can undergo phosphorylation. Specific chemical cross-linking experiments in 70z mouse cells have shown Hsp60 to be associated with $P21^{ras}$, which is a plasma membrane protein involved in signal transduction (Ikawa and Weinberg, 1992). This finding implies that Hsp60 might be involved in signal transduction events at the cell surface. Hsp60 has also been shown to interact with prion protein PrP^{c} (Edenhofer et al., 1996) and HIV transmembrane glycoprotein gp41 (Speth et al., 1999).

In another study Jones *et al.* (1994) have implied Hsp60 to be involved in cell surface amino acid transport. In this study, CHO-K1 mutant that show an increase of A system of amino acid transport were found to exhibit a concomitant increase in the amount of Hsp60 associated with the cell surface.

Mitochondrial Hsp70

Mitochondrial Hsp70 was identified as a peptide binding protein PBP 72/74, because it was implied to function in antigen presentation (Domanico et al., 1993; Dahlseid et al., 1994). mHsp70 has also been identified as mortalin because of its implicated role in cell senescence (Wadhwa et al., 1993a; Wadhwa et al., 1994). Both these roles assigned to mHsp70 suggest an extramitochondrial role for it. Furthermore, using immunoelectron microscopy our lab has shown mHsp70 to be localized at the plasma membrane, cytoplasmic vesicles and in unidentified cytoplasmic granules of BS-C-1 cells (Singh et al., 1997). The antibodies used in this study were specific to mHsp70 and showed no cross reactivity to cytosolic Hsp70.

Presence of Hsp60 and mHsp70 at extramitochondrial sites could be due to leaky import or mistargeting. Evidence against this comes from nonactin experiments. The potassium ionophore nonactin disrupts membrane potential and inhibits protein import. CHO cells treated with nonactin, accumulate only precursor Hsp60, while in untreated cells, only mature Hsp60 is detectable (Soltys and Gupta, 1996). This suggests that maturation of precursor protein occurs only after import into mitochondria and that the levels of precursor protein under normal conditions are too low to be detected. This suggests that the labeling seen at extramitochondrial locations are due to reaction of the antibody with the mature forms of Hsp60 and mHsp70, which are probably exported to these locations. Furthermore, microsequencing results from numerous studies have indicated that it is the mature form of Hsp60 that is present at extramitochondrial locations (Ikawa and Weinberg, 1992; Jones et al., 1994; Khan et al., 1998).

Hsp10 / Cpn10

Cpn10 is a necessary co-factor for Hsp60 and is thought to bind to one end of Hsp60 and act as a cap to prevent substrate release during folding (Weissman et al., 1994). Cpn10 has also been identified as being identical to early pregnancy factor (EPF), which appears in maternal serum within 24 hours of fertilization (Cavanagh and Morton, 1994). EPF has been shown to be involved in the regulation of maternal immune system at the early stages of pregnancy namely the first half of gestation. It is thought to have an inhibitory effect on immunocompetence of maternal lymphocytes, thus enabling the survival of the fetus (Cavanagh and Morton, 1994; Noonan et al., 1979). Immunosupressive nature of EPF is supported by the observation that serum from

pregnant animals is able to inhibit lymphocyte response in various assays (Morton et al., 1974). Further evidence for this is provided by the fact that loss of embryo due to spontaneous or therapeutic abortions is associated with lack of EPF activity (Rolfe, 1982) and also, the mice that were administered with EPF antibody before implantation showed a decreased efficiency of implantation (Athanasas-Platsis et al., 1991).

EPF has also been shown to be present in serum during renewal of tissue, regeneration of liver (Quinn et al., 1994) and development of cancer (Quinn and Morton, 1992), thus indicating that it is involved in growth regulation as well. In vitro, it has been shown that EPF activity is coincident with cellular growth and absence of EPF activity is coincident with growth arrest or differentiation (Quinn et al., 1990). Like many other growth factors, EPF is present in platelets and this could imply a function in inflammation and wound healing (Cavanagh and Morton, 1994).

Aspartate Aminotransferase

Mitochondrial aspartate aminotransferase (mAspAT) is synthesized wth a N-terminal mitochondrial targeting sequence which is cleaved upon import into the matrix. Mitochondrial AspAT is an important enzyme in amino acid metabolism (Christen and Metzler, 1985) and is identical to a fatty acid binding protein ($FABP_{pm}$) isolated from plasma membranes of several cell types (Berk et al., 1990; Stump et al., 1993). $FABP_{pm}$ is a transporter of long chain free fatty acids (Schwieterman et al., 1988). Transfection of 3T3 cells that do not express mAspAT with mAspAT cDNA led to saturable fatty acid uptake (Isola et al., 1995). Also, the surface and mitochondrial localization of this protein was confirmed with immunofluorescence microscopy in these cells.

P32

P32 is a mitochondrial protein synthesized with a N-terminal targeting sequence which is cleaved upon import into the matrix (Muta et al., 1997). Immunofluorescence microscopy has identified it to be localized mainly within the mitochondria. It is involved in numerous nuclear functions and is also a cell surface receptor for complement component C1q (Ghebrehiwet et al., 1994), in B cells, neutrophils and mast cells. Using immunoelectron microscopy we have shown P32 protein to be present in mitochondria and at extramitochondrial locations such as zymogen granules, endoplasmic reticulum, nucleus and cell surface in various tissues (Soltys et al., 2000).

Tumor Necrosis Factor Receptor Associated Protein 1

Tumor necrosis factor receptor associated protein 1 (TRAP-1) is a distant homolog of Hsp90 family of molecular chaperones (Song et al., 1995). It acts as a partner for a variety of proteins involved in a wide range of functions such as cell signaling, tumorogenesis, immune response and apoptosis (Heller and Kronke, 1994). It has also been identified as a retinoblastoma binding protein (Chen et al., 1996). Upon cloning TRAP-1 was found to be a mitochondrial matrix protein with a N-terminal targeting sequence (Felts et al., 2000). Immunoelectron microscopy revealed TRAP-1 to be present in mitochondria and at various extramitochondrial sites in rat tissues. In the pancreas, labeling was observed in mitochondria, nucleus, zymogen granules, insulin secretory granules and cell surface. Labeling was also observed in heart nuclei and endothelial cells lining blood vessels (Cechetto and Gupta, 2000).

Fumarase

In yeast, both cytosolic and mitochondrial fumarase are encoded by a single gene FUM1 (Wu and Tzagoloff, 1987). All of the fumarase synthesized in the cytosol has a N-terminal targeting sequence. It is the mature form of fumarase that is present in cytosol and matrix. Before distribution, fumarase is co-translationally imported and once the N-terminal presequence is cleaved, it can either be fully translocated into the matrix or end up in the cytosol by a mechanism that has not been established yet. This way 70-80% and 20-30% of the fumarase end up in cytosol and mitochondrial matrix respectively (Stein et al., 1994). Two models have been proposed for the mechanism by which fumarase ends up in cytosol (Knox et al., 1998): (i) The fumarase is fully translocated into the matrix folded and then exported out by an unknown mechanism. (ii) After the cleavage of the presequence it could reverse direction through retrograde movement and end up in the cytosol. Retrograde movement could either be due to mHsp70 not binding to the translocating protein or the rapid folding of domains would prevent vectorial translocation into the matrix and cleavage of presequence would result in slippage of the protein into the cytosol.

Mitochondrial Intermembrane Space Proteins

Cytochrome c

Cytochrome c is an essential part of the electron transport chain of mitochondria and is known to localize in the mitochondrial intermembrane space (Lehninger et al., 1993). Recently, it has been shown that following apoptotic stimuli, which is characterized by the permeabilization of the outer membrane, cytochrome c is relocalized to the cytosol. Presence of cytochrome c in the cytosol, leads to the activation of

caspases followed by cell death (Green and Reed, 1998; Desagher and Martinou, 2000; Brenner and Kroemer, 2000). Cytochrome c is nuclear encoded and is synthesized as apo-cytochrome c (- heme). An interesting feature about cytochrome c is that it does not have a cleavable pre-sequence and its import into mitochondria is lipid mediated. Once in the intermembrane space, apo-cytochrome c is converted to holo-cytochrome c by the covalent addition of heme by cytochrome c heme lyase (Neupert, 1997).

Localization of cytochrome c using immunoelectron microscopy in a variety of normal rat tissues (viz., adrenal gland, cerebellum, cerebral cortex, heart, kidney, liver, pituitary, pancreas, skeletal muscle, spleen and thyroid), revealed that localization was primarily in mitochondria. However, substantial amount of cytochrome c was also detected at extramitochondrial sites in the pancreas and pituitary. In the pancreas, labeling was found in zymogen granules and acinar lumen, and in the pituitary, labeling was found in growth hormone granules (Soltys et al., 2001). At this point, it is not known whether it is the apo- or holo-cytochrome c that is present at these extramitochondrial sites. It is important to note that no labeling was seen in the cytosol where it can lead to cell death via activation of caspases. This work has led to the suggestion that protein translocation from mitochondria to other specific compartments, occur under normal conditions and the disruption of these pathways could lead to release into the cytosol and lead to apoptosis (Soltys et al., 2001).

Apoptosis Inducing Factor

Apoptosis inducing factor (AIF) is a flavoprotein which is involved in oxidoreductase function in the mitochondrial intermembrane space. It is synthesized with

a N-terminal mitochondrial targeting sequence, which is cleaved upon import into mitochondria (Susin et al., 1999a). AIF has been shown to have a second, independent apoptogenic function. It has been shown that after the induction of apoptosis, AIF translocates to the nucleus (Susin et al., 1996). The subcellular localization of AIF in normal and apoptotic cells has been confirmed by immunofluorescence microscopy (Daugas et al., 2000b).

Recombinant AIF, independent of cytosolic extracts, cause chromatin condensation in isolated nuclei and causes large scale DNA fragmentation. Microinjection of AIF into cytoplasm of normal cells, leads to the loss of mitochondrial transmembrane potential, release of apoptogenic proteins and the condensation of nuclear chromatin (Susin et al., 1996). The membrane permeabilization effects of AIF requires a cytosolic heat sensitive factor (Susin et al., 1999a). These effects induced by AIF are not blocked by the wide ranging caspase inhibitor known as Z-VAD.fmk, which suggests that AIF, unlike cytochrome c, acts in a caspase-independent manner (Susin et al., 1999b).

Various mitochondrial proteins have been shown to be present at extramitochondrial sites in certain cell types and tissues. Some of these proteins at these extramitochondrial locations, have been shown to be involved in novel functions which are different from their functions in mitochondria. The mechanisms by which these proteins might be transported to these extramitochondrial sites still remains enigmatic. Therefore, the role of mitochondria and mitochondrial proteins in normal cellular processes, needs to be re-examined.

1.7 Project Goals

As we have seen, a large number of mitochondrial proteins have already been shown to function at extramitochondrial locations. The goal of the study is to examine the subcellular localization of mitochondrial proteins at extramitochondrial locations, with Cpn10, mAspAT, and mitochondrially encoded cytochrome c oxidase subunit I and II (COX I / II). Of these, the first two, Cpn10 and mAspAT, have already been shown to have extramitochondrial functions.

Traditionally, localization of a given protein to mitochondria is based on immunofluorescence microscopy, presence of a N-terminal targeting sequence and cell fractionation studies. Using these techniques, the presence of small amounts of mitochondrial proteins at extramitochondrial sites cannot be resolved. Based on extensive work carried out in the lab, immunoelectron microscopy, with the aid of specific antibodies has been shown to be most reliable in resolving proteins at mitochondrial and extramitochondrial sites. Thus, immunoelectron microscopy was exclusively used in the localization studies. This technique becomes very informative for proteins which are localized in specific compartments such as insulin secretory granules, zymogen granules, growth hormone granules and peroxisomes. Since not all cells contain these specific compartments, localization studies were carried out on various rat tissues and cell types. A major determinant of the validity of immunoelectron microscopic studies is the specificity of the antibodies used. Thus, specific and well characterized antibodies were either obtained through collaboration or purchased from commercial sources, where their specificity has been established by western blotting,

immunofluorescence microscopy and immunoprecipitation. Furthermore, the specificity of the antibody (where possible) was demonstrated by adsorbing the antibody with recombinant antigen, which abolished labeling.

The reason why Cpn10, mAspAT and COX I / II were chosen to be studied, is as follows: Cpn10 is a co-factor for Hsp60 and has also been identified to be identical to extramitochondrial EPF, which has been shown to be an immunosuppresant, a growth factor and implied in inflammation and wound healing (Cavanagh and Morton, 1994; Morton et al., 1974; Quinn et al., 1990). Since Hsp60 has already been shown to be at a variety of extramitochondrial locations such as peroxisomes, cytosolic vesicles, mature insulin secretory granules, zymogen granules and growth hormone granules (Soltys and Gupta, 2000), it would be informative to see if Cpn10 is localized to these compartments as well. Localization of both Hsp60 and Cpn10 in these compartments could mean that they function as molecular chaperones. Except for two localization studies of Cpn10 in malignant human colonic tissue (Somodevilla-Torres et al., 2000) and rat pancreatic acinar cells (Vélez-Granell et al., 1994), no detailed subcellular localization of Cpn10 in a variety of normal tissues has been done. It is important to note that the latter study employed a polyclonal antibody raised against the bacterial homolog of *Chromatium vinosum*.

Mitochondrial AspAT has been identified to be identical to fatty acid binding protein, which is a plasma membrane protein involved in the transport of long chain free fatty acids (Stump et al., 1993; Schwieterman et al., 1988). In the past, work on the subcellular distribution of mAspAT, has focused almost exclusively on the liver and the

extent to which mAspAT is present at extramitochondrial locations in other tissues is not known.

The subcellular localization of COX I and II is of great interest to us because they are encoded by the mitochondrial DNA and, also transcribed and translated within the mitochondria. When mitochondrial proteins encoded by the nucleus are found at extramitochondrial locations, it has proven difficult to rigorously exclude the possibilities that the results are not due to leaky mitochondrial import, mistargeting of the protein, alternate splicing, transcription or translation initiation. Hence, if the results from mitochondrial DNA encoded proteins show that these proteins are present at extramitochondrial locations, it would provide compelling evidence that proteins can, in fact, exit the mitochondria. Peptides derived from mitochondria encoded proteins have already been shown to be present at extramitochondrial locations. Mitochondrially transmitted factors (MTF) are present on the cell surface as minor histocompatibility antigen and are encoded by mitochondrial DNA (Fischer-Lindahl et al., 1991; Dabhi and Lindahl, 1995). Cell surface expression of MTF is inhibited by chloroamphenicol which specifically blocks mitochondrial protein synthesis. In humans and mouse, MTF is derived from mitochondria encoded NADH dehydrogenase subunit I (Loveland et al., 1990) while in rat, it is derived from mitochondria encoded ATP synthase subunit 6 (Bhuyan et al., 1997).

Chapter 2.0 :

Materials and Methods

2.1 Reagents and Chemicals

Nitrocellulose sheet and percoll were purchased from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec). Glycerol, glycine, maleate, β-mercaptoethanol and sucrose were purchased from BDH Chemicals Limited (Toronto, ON). 4-Chloro-1-naphthol was purchased from Bio-Rad Laboratories (Mississauga, ON). Acrylamide, bis-acrylamide and Tris-HCl were purchased from BioShop Canada Inc. (Burlington, ON). Bovine serum albumin (BSA, Fraction V) was purchased from Boehringer Mannheim Canada (Laval, Quebec). Sodium pentobarbital was purchased from BIMEDA-MTC Animal Health Inc. (Cambridge, ON). Ethanol was purchased from Commercial Alcohols Inc. (Burlington, ON). Hydrogen peroxide and methanol were purchased from EM Science (Gibbstown, NJ). Ammonium persulphate, fetal calf serum (FCS), α-minimal essential medium (α-MEM) and TEMED were purchased from Life Technologies (Burlington, ON). Uranyl acetate was purchased from Marivac Limited (Halifax, Nova Scotia). LR Gold resin was purchased from Polysciences (Warrington, PA). Sodium dodecyl sulphate (SDS) was purchased from Sangon Limited Canada (Toronto, ON). Benzamidine, Coomassie blue, dithiothreitol, EGTA, MES, MOPS, paraphenyldiamine, PMSF and soybean trypsin inhibitor were purchased from Sigma (St Louis, MO). Paraformaldehyde was purchased from TAAB Laboratories Equipment Limited (Berks., England).

2.2 Antibodies

The preparation and characterization of rabbit polyclonal antibodies against the C-terminal peptide of human Cpn10 (residues 77-101) and, mouse monoclonal (MAbII-13) and rabbit polyclonal (P_1-3) antibodies against human recombinant Hsp60 has been previously described (Somodevilla-Torres et al., 2000; Singh and Gupta, 1992; Singh and Gupta, 1992). Rabbit polyclonal antibodies to purified rat liver mAspAT were raised and characterized as described previously (Stump et al., 1993). Mouse monoclonal antibodies against human cytochrome c oxidase subunit I and II were purchased from Molecular Probes (Eugene, OR). Other antibodies that were commercially obtained include, rabbit polyclonal antibody against human α-amylase (Sigma; St Louis, MO), rabbit polyclonal antibody against sheep growth hormone (ICN; Montreal, Quebec, Canada), sheep polyclonal antibody against bovine catalase (Serotec; U.K.) and mouse monoclonal antibody against human insulin (Sigma; St Louis, MO). For immunoelectron microscopy, secondary goat anti-rabbit, goat anti-mouse and goat anti-sheep IgG 20 nanometer (nm) gold conjugate antibodies were purchased from British BioCell (Hornby, Ontario). For immunofluorescence microscopy, secondary Texas red or fluorescein conjugated goat anti-mouse or goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Labs (West Grove, PA). For western blots horseradish peroxidase conjugated goat secondary antibodies against rabbit IgG were purchased from Bio-Rad Laboratories (Mississauga, ON).

2.3 Cell Lines / Tissue Culture / Double Immunofluorescence Microscopy

Double immunofluorescence labeling of cultured cells was carried out as described earlier (Gupta and Dudani, 1987), with some modifications. African green monkey kidney cells, BS-C-1 cells (ATCC CCL-26) were grown in α-MEM medium, supplemented with 5% fetal calf serum at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells were then grown on sterile coverslips for 48 hours after which they had attained a flattened morphology. Coverslips were briefly washed in Tris buffered saline (TBS) (0.9% NaCl, 10 mM Tris-HCl, pH 7.5) and the cells were fixed in ice cold methanol at –20°C for 20 minutes and then briefly washed in TBS. Following fixation cells were blocked with 50% normal goat serum in 0.1 M Tris-HCl (pH 7.5) and then incubated with 1:100 (10 μg/ml) diluted Cpn10 antibody for 45 minutes at 37°C in a humidified chamber. After the primary incubation, the cover slips were washed with three changes of TBS for 15 mintues. The cells were then labeled with the secondary fluorescein conjugated goat anti-rabbit IgG diluted 1:100 for 45 minutes at 37°C in a humidified chamber. A second labeling was carried out with 1:100 (ascites) diluted mouse monoclonal Hsp60 antibody followed by labeling with secondary texas red conjugated goat anti-mouse IgG diluted 1:100. The cover slips were washed in TBS and then briefly in water. The coverslips were mounted on a slide containing a small drop of mounting medium (1mg/ml paraphenyldiamine, in 90% glycerol and 100 mM Tris, pH 8.0). The labeled cells were examined as described earlier (Gupta and Dudani, 1987).

The other antibodies used were diluted as follows, 1:40 rabbit polyclonal Hsp60 antibody, 1:40 rabbit polyclonal mAspAT antibody, 1:100 (10 μg/ml) mouse monoclonal cytochrome c oxidase subunit I and II antibody.

2.4 Immunoelectron Microscopy

Spraque Dawley rats (Charles River Labs, Wilmington, MA) were anaesthetized with sodium pentobarbital and perfusion fixed with 4% paraformaldehyde in 100mM sodium phosphate buffer, pH 7.4, freshly made before use. Tissues were excised, kept on ice, cut into 1mm cubes, washed 3 times with 0.1M sucrose, 0.1M maleate buffer, pH 6.0, postfixed for 30 minutes in 1% uranyl acetate in the same buffer and then followed by 4% uranyl acetate in 50% ethanol for 30 minutes. Serial dehydration was carried out with ethanol in the order of 70%, 90%, 95% and 100%, with each step lasting 10 minutes. The rest of the procedure for embedding and sectioning of cells in LR Gold resin was done as described earlier (Soltys and Gupta, 1996).

For labeling of rat tissue sections embedded in LR Gold resin the sections were first wetted in 0.1M Tris-HCl, pH 7.5 (carrier buffer) for 10 minutes and then were preabsorbed at room temperature with 20% fetal calf serum in carrier buffer. Sections were then incubated with 1:40 (25 μg/ml) diluted Cpn10 antibody in carrier buffer for 2 hours at 37°C, in a humidified chamber. For the antibody preadsorption controls, Cpn10 antibody was incubated with nitrocellulose strips saturated with purified recombinant human Cpn10 protein for 2 hours at 37°C, before carrying out the labeling. Following this, the sections were washed with 3 changes of 1% BSA for 10 minutes. The sections were then incubated with the secondary goat anti-rabbit IgG 20 nm gold conjugate at a 1:10 dilution in carrier buffer for 4 hours at 37°C, in a humidified chamber. Following this, sections were washed with 0.5M KCl in carrier buffer and quickly rinsed in water.

Sections were then stained with 4% uranyl acetate in 25% ethanol for 20 mintues, rinsed briefly with 25% ethanol and then air dried. Sections were examined at 80 kV with a JEOL 1200 EX transmission electron microscope.

The other antibodies were diluted as follows, 1:30 rabbit polyclonal mAspAT antibody, 1:40 (25 μg/ml) mouse monoclonal cytochrome c oxidase subunit I and II antibodies, 1:500 rabbit polyclonal α-amylase antibody, 1:1000 rabbit polyclonal growth hormone antibody, 1:500 sheep polyclonal catalase antibody and 1:500 mouse monoclonal insulin antibody. All the secondary antibodies were used at 1:10 dilution.

2.5 Purification of Zymogen Granules from Bovine Pancreas

Zymogen granules were isolated from bovine pancreas with modifications to previously published protocol (Thevenod et al., 1990). Fifty grams of bovine pancreatic tissue was immersed in ice cold homogenization buffer containing, 250 mM sucrose, 50 mM MOPS (pH 7.0), 0.1 mM $MgSO_4$, 0.1 mM EGTA, 1 mg/ml defatted BSA, 0.2 mM PMSF (added just before use from a 100 mM stock solution in ethanol), 1 mM benzamidine and 0.01% soybean trypsin inhibitor. The pancreas was cut into small pieces with a scissor, while discarding any fat and connective tissue. The small pieces of pancreas were suspended in 15% (wt/vol) of ice cold homogenization buffer and homogenized using a Tissuemizer for 10s at 1, 900 rpm. Then the tissue was homogenized using a motorized Teflon glass homogenizer (10 strokes at about 500 rpm). The homogenate was then centrifuged for 10 minutes at 500 x g at 4°C, to remove unbroken cells, debris and nuclei. The resulting supernatant was further centrifuged for

15 minutes at 1, 500 x g at 4°C. The resulting brownish layer of mitochondria on top of the white zymogen granule pellet was gently removed by swirling two times with 1ml of homogenization buffer and was saved for western blotting. The crude zymogen granule pellet was resuspended in Percoll-sucrose-MES gradient (40% Percoll, 250 mM sucrose, 50 mM MES, pH 6.2, 2 mM EGTA, 0.2 mM $MgSO_4$, 1 mg/ml defatted BSA, 0.2 mM PMSF, 1 mM benzamidine and 0.01% soybean trypsin inhibitor), at 20 ml/g of tissue and was centrifuged at 20, 000 x g for 20 minutes at 4°C. Zymogen granules were obtained as a white band at the bottom of the tube. The zymogen granules were diluted ten fold in wash buffer (250 mM sucrose, 50 mM MES, pH 6.2, 2 mM EGTA, 0.2 mM $MgSO_4$, 1 mg/ml defatted BSA, 0.2 mM PMSF, 1 mM benzamidine and 0.01% soybean trypsin inhibitor) and washed two times and loaded again onto another Percoll-sucrose-MES gradient, centrifuged, washed twice and eventually resuspended in 125 mM Tris-HCl, pH 6.8 for western blot analysis.

2.6 Processing of Purified Bovine Zymogen Granules for Electron Microscopy

Immediately after purification, zymogen granules were fixed in 4% paraformaldehyde in 125 mM MES buffer (pH 6.5) overnight at 4°C. Granules were then post-fixed in uranyl acetate, dehydrated in ethanol, embedded in LR Gold resin and sectioned as described before (Soltys and Gupta, 1996). The purity of these zymogen granules as determined by electron microscopy is shown in Figure 1.

2.7 Gel Electrophoresis and Western Blots

Equal amounts of protein samples either from purified zymogen granules or mitochondria were electrophoresed in 10% or 15% SDS-PAGE as described previously (Gupta and Dudani, 1987; Soltys and Gupta, 1996). The proteins were either visualized by Coomassie blue staining or transferred electrophoretically from polyacrylamide gels to nitrocellulose sheets. Following the transfer, the blot was blocked with 3% BSA in TBS, for 1 hour at 37°C. This was also used as a carrier buffer for all primary and secondary antibodies. The blots were incubated with primary antibodies diluted, 1:2000 rabbit polyclonal antibody to α-amylase or 1:1000 affinity purified rabbit polyclonal antibody to Cpn10 (1 μg/ml) or 1:1000 rabbit polyclonal antibody to mAspAT, for two hours at room temperature with gentle rocking. Secondary antibodies conjugated to horseradish peroxidase and directed against rabbit IgG were used to visualize the primary antibodies bound to the blot, using colour development with 4-chloro-1-naphthol.

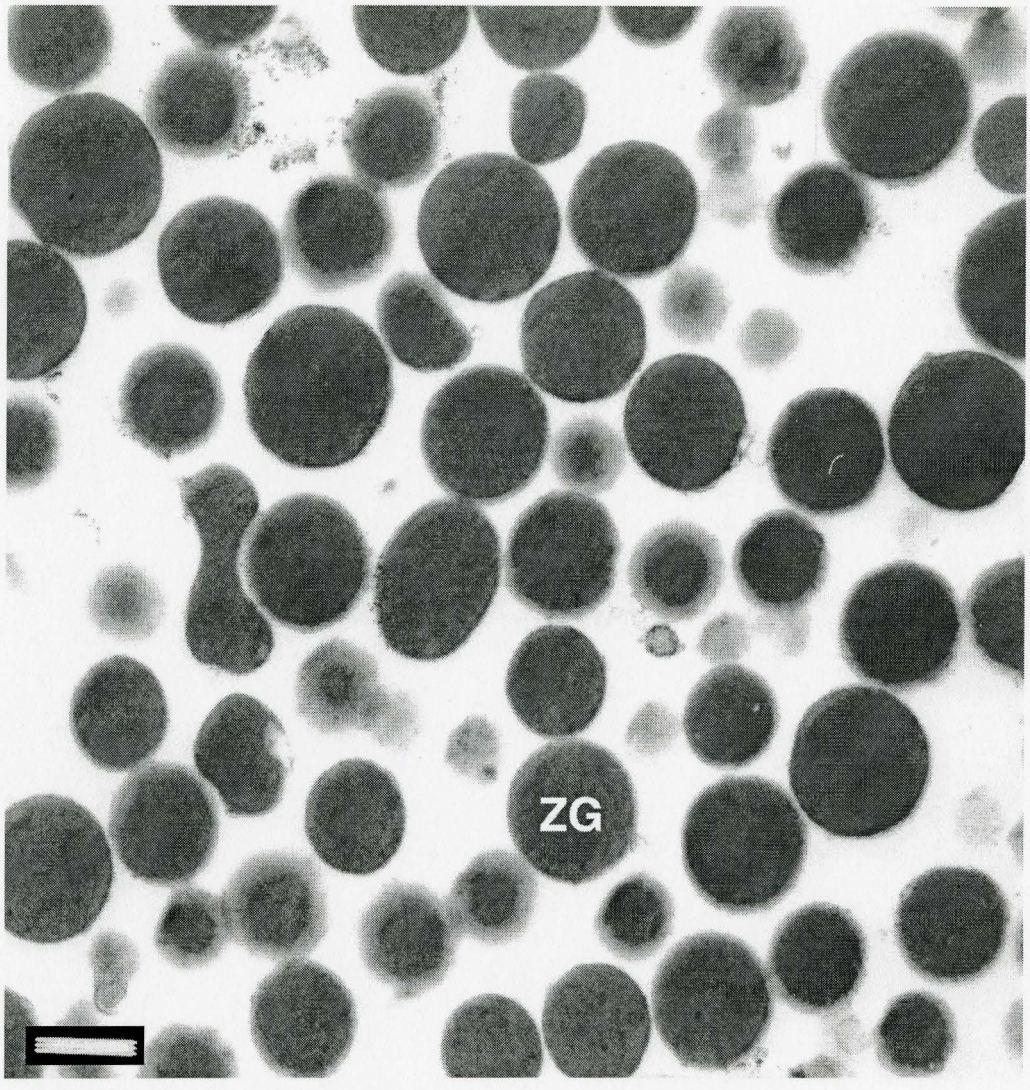

Figure 1: An electron micrograph of purified electron dense zymogen granules from bovine pancreas. Note the heterogeneity in size and the purity of these granules. ZG, zymogen granules. Bar = 500 nm.

Chapter 3.0:

Immunoelectron Microscopy Provides Evidence for the Presence of Mitochondrial Chaperonin 10 in Red Blood Cells and a Variety of Secretory Granules

3.1 Abstract

Chaperonin 10 (Cpn10) is a co-chaperonin for Hsp60 and has also been identified as being identical to early pregnancy factor (EPF), which is an immunosuppressive/growth factor found in maternal serum. In this study we have used immunogold electron microscopy to study the subcellular localization of Cpn10 in cultured cells and in a variety of rat tissues sections embedded in LR Gold resin employing a polyclonal antibody raised against the C-terminal end of human Cpn10. Immunofluorescence labeling of BS-C-1 African monkey kidney cells with this antibody showed predominant labeling of only mitochondria. In immunogold labeling studies, the Cpn10 antibody also showed strong labeling of mitochondria in various rat tissues examined including the liver, heart, pancreas, kidney, anterior pituitary, salivary gland, thyroid and adrenal gland. However, in addition to the mitochondrial labeling, in a number of rat tissues, strong and highly specific labeling with the Cpn10 antibody was also observed at a number of extramitochondrial locations, including pancreatic zymogen granules, red blood cells, growth hormone granules in anterior pituitary and secretory granules in PP pancreatic islet cells. Cpn10 labeling was also observed in purified bovine zymogen granules. The observed labeling with the Cpn10 antibody, both within mitochondria as well as in various other compartments, was abolished upon omission of the primary antibody or upon preadsorption of the primary antibody with the purified recombinant human Cpn10 protein. These results provide evidence that similar to a number of other recently described mitochondrial proteins (viz., Hsp60, tumor necrosis factor receptor associated protein-1 (TRAP-1), P32 protein and cytochrome c), Cpn10 is

also found at a variety of specific extramitochondrial sites in normal rat tissue. These results raise fundamental questions concerning the possible functional roles of mitochondria and mitochondrial resident proteins in diverse celluar processes in different compartments and the mechanism by which these proteins reach such sites. The presence of mitochondrial proteins at extramitochondrial sites has also important implications regarding the role of mitochondria in different diseases.

3.2 Introduction

Chaperonin 10 (Cpn10) or heat shock protein 10 (Hsp10) belongs to a subclass of molecular chaperones called Group I chaperonins that are found in bacteria, mitochondria and plastids and are essential cellular proteins under all cellular conditions (Lindquist and Craig, 1988; Georgopoulos and Welch, 1993; Gupta, 1995). Cpn10 is a mitochondrial matrix protein that is synthesized with a N-terminal targeting sequence, but unlike most other matrix proteins this targeting sequence is not cleaved upon import into the mitochondria (Ryan et al., 1995). Cpn10 is a co-chaperonin for Hsp60 and the bacterial counterparts for Cpn10 and Hsp60 are the GroES and GroEL proteins (Hartl, 1996). GroES/Cpn10 is thought to bind to one end of GroEL/Hsp60 and act as a cap to prevent substrate release during the ATP-hydrolysis catalyzed folding process (Weissman et al., 1994). The GroEL-GroES machinery acts as a double toroidal ring, whereas the Hsp60-Cpn10 machinery acts as a single toroidal ring (Picketts et al., 1989; Viitanen et al., 1992). In view of the established function of Cpn10 within mitochondria, it was a very surprising discovery that a protein previously identified as early pregnancy factor (EPF), which appears in maternal serum within 24 hours of fertilization, upon subsequent characterization was found identical to the Cpn10 protein (Cavanagh and Morton, 1994).

The evidence indicating that EPF purified from human platelets was identical to the Cpn10 protein is derived based on a number of different observations: (i) The amino acid sequence of three different fragments covering more than 70% of the EPF protein showed complete identity with the human Cpn10 protein. (ii) Purified rat Cpn10 was found to be as active in the EPF bioassay as the platelet derived EPF, and this activity

could be neutralized by a monoclonal antibody to EPF. In contrast, to the mammalian Cpn10, bacterial GroES was not active in the bioassay, providing evidence of specificity. (iii) In the presence of ATP, EPF, similar to Cpn10 formed stable complex with Hsp60 that coeluted from gel-filteration column. Further, the immobilized-Hsp60, in the presence of ATP but not in its absence, was able to remove all EPF-activity from pregnancy serum, providing further evidence of a specific interaction between these proteins (Cavanagh and Morton, 1994).

In our recent work we have shown that Hsp60 which is the functional partner of Cpn10 and is a well-studied mitochondrial protein by various criteria, is present at a variety of specific sites and compartments, outside of mitochondria (Cechetto et al., 2000; Soltys and Gupta, 1996). In view of this and the observed identity of Cpn10 with EPF, it is of much interest to examine in detail the subcellular localization of Cpn10/EPF in different tissues and cell types. Previously, using immunohistochemical techniques it was inferred that Cpn10/EPF is present in the mitochondria and also at extramitochondrial sites such as the cytoplasm, secretory membranes and secretory glandular spaces in malignant human colonic tissue (Somodevilla-Torres et al., 2000). However, the immunohistochemical technique has only limited resolution and based on this the subcellular distribution/localization of an antigen cannot be accurately inferred. Therefore, in the present work we have used high resolution immunogold electron microscopy technique to determine the subcellular localization of Cpn10 in a variety of normal rat tissue sections embedded in LR Gold resin. In a number of tissues examined, Cpn10 was mainly within the mitochondria. However, in many other tissues in addition

to mitochondria strong and specific labeling with Cpn10 antibodies was also observed at a variety of extramitochondrial sites including pancreatic zymogen granules, red blood cells, anterior pituitary growth hormone granules and secretory granules in PP pancreatic islet cells. Cpn10 reactivity was also seen in purified bovine zymogen granules. The presence of Cpn10 at these extramitochondrial sites, raises important questions concerning its function at these locations and how it possibly reaches these sites.

3.3 Results

The Cpn10 antibody used in the present work is an affinity purified polyclonal antibody raised against the C-terminal peptide of human Cpn10 (residues 77-101). This antibody reacts specifically with Cpn10 as determined by sandwich ELISA, immunoblotting, immunoprecipitation and immunohistochemistry (Somodevilla-Torres et al., 2000). The Cpn10 and Hsp60 antibodies were employed for double immunofluorescence labeling of cultured African green monkey kidney, BS-C-1 cells. The punctate and bead-string shaped labeling pattern observed was typical of mitochondrial staining (Figure 1), for both Cpn10 and Hsp60. Furthermore, the labeling pattern observed with Cpn10 and Hsp60 show complete overlap, demonstrating that the Cpn10 antibodies are infact labeling mitochondria. There was no indication of any labeling outside of mitochondria from this experiment.

The subcellular localization of Cpn10 was next studied in normal rat tissues and cultured CHO cells by means of high resolution immunogold labeling microscopy. In CHO cells sections embedded in LR Gold resin, the labeling was restricted primarily within mitochondria, as seen by the distribution of 20 nm gold particles (not shown). There was virtually no labeling seen in the nucleus, endoplasmic reticulum and cytosol. Figure 2A and 2B show the labeling patterns observed in rat liver and kidney sections. In rat liver (Figure 2A) the reactivity due to Cpn10 antibody was seen mainly in the mitochondria with weak background level of labeling seen in the nucleus, endoplasmic reticulum and cytosol. Likewise, in the distal convoluted tubule regions of the kidney strong labeling was seen within mitochondria and a low background level of labeling in

other compartments (Figure 2B). It is interesting to note that the labeling seen within the individual mitochondria in Figure 2B is variable with certain regions more intensely labeled. Similar strong and specific labeling of mitochondria with Cpn10 antibody was observed in other tissues such as heart, proximal convoluted tubule region of the kidney, salivary gland, thyroid and adrenal gland.

In contrast to the above tissues, subcellular localization of Cpn10 in rat pancreatic acinar cell was not exclusive to mitochondria, specific labeling was also observed in zymogen granules (Figure 3A). In this case, the labeling density due to Cpn10 within zymogen granules was comparable or slightly higher than that seen in mitochondria. Cpn10 in pancreatic acinar cells is localized almost exclusively within zymogen granules and mitochondria with virtually no labeling seen in endoplasmic reticulum, cytosol or the nucleus. To determine whether the observed labeling of zymogen granules with the Cpn10 antibody was specific, the antibody was preadsorbed with purified recombinant human Cpn10 antigen and then used to label pancreatic rat tissue sections. The results of these studies presented in Figure 3B, show that preadsorption of the antibody with the purified recombinant protein abolishes all of the labeling seen in mitochondria, zymogen granules and other compartments. As a control, Cpn10 antibodies preadsorbed with BSA does not abolish labeling in zymogen granules and mitochondria (not shown). Also, omitting the primary Cpn10 antibody in the labeling abolished labeling, showing that the secondary immuno-gold markers do not bind to the LR Gold resin (Figure 4). These results provide evidence that labeling seen in zymogen granules is specifically due to a Cpn10 cross-reactive protein. In contrast to

Cpn10, subcellular localization of α-amylase in rat pancreatic acinar tissue showed labeling all along the secretory pathway, which included ER, Golgi, condensing vacuoles, zymogen granules and acinar lumen (Figure 5). No mitochondrial labeling is seen.

To confirm the presence of Cpn10 in pancreatic zymogen granules, zymogen granules were purified from bovine pancreas. The reason for using bovine pancreas instead of rat pancreas was because the yield per animal in the latter was too low. The purity of the purified bovine zymogen granules was determined by electron microscopy and was free of mitochondria. Figure 6, shows the labeling of Cpn10 within purified bovine zymogen granules, with very little background labeling seen in the resin. The purified bovine granules were identified as zymogen granules due to their specific reactivity with α-amylase antibody (not shown).

Platelets have previously been shown to be a rich source of Cpn10 (Cavanagh and Morton, 1994). Accordingly, Cpn10 in this work was also localized to red blood cells found within blood vessels in rat heart (Figure 7A) and pancreas (Figure 7B). Intense labeling due to Cpn10 is seen within the red blood cells which are identified due to their unique morphological biconcave shape as in Figure 7A. The gold labeling seen in the pericapillary space, basal lamina and lumen of the blood vessel can be attributed to background.

In the rat anterior pituitary tissue sections, Cpn10 was localized to mitochondria and growth hormone granules, with background level labeling seen in the nucleus and cytoplasm as shown in Figure 8. These granules have been identified as growth hormone granules due to their reactivity with an antibody to the growth hormone (not shown). The

labeling pattern within the granules is heterogeneous with certain regions being more intensely labeled than others. The labeling intensity within growth hormone granules is at least comparable and possibly much higher than that seen within mitochondria.

Subcellular localization of Cpn10 was also examined in the pancreatic islet cells. The Cpn10 reactivity in this case was found in all mitochondria as well as PP cell secretory vesicles. In Figure 9 the left half of the micrograph corresponds to a PP cell with dense granules and the right half corresponds to an insulin secretory cell, containing insulin secretory granules. Cpn10 is localized to mitochondria and PP cell secretory vesicles with no appreciable labeling seen in insulin secretory vesicles, nucleus and cytoplasm.

3.4 Discussion

Results presented in this chapter provide strong and unequivocal evidence for the presence of Cpn10 within mitochondria from all cells and tissues, which is in accordance with the known function of Cpn10 as a co-chaperone for the mitochondrially-localized Hsp60 protein in the protein folding process. The mitochondrial localization of Cpn10 is also supported by immunofluorescence labeling studies. However, our results also provide evidence that in addition to mitochondria, strong reactivity to Cpn10 antibody is also seen at a variety of specific extramitochondrial locations in different rat tissues. The sites where strong Cpn10 reactivity has been observed include the red blood cells from heart and pancreas, zymogen granules from pancreatic acinar cells, PP granules from pancreatic islet cells, and growth hormone granules from anterior pituitary. The Cpn10 labeling intensity in these compartments was at least comparable (if not higher) than that seen in mitochondria. In contrast, the labeling in cytoplasm, nucleus and endoplasmic reticulum was very weak and generally at background levels. The observed labeling in all these cases was shown to be specific by a number of criteria: (i) The labeling in both mitochondria and all these cells/compartments was completely abolished upon preadsorption of the Cpn10 antibody with purified human recombinant Cpn10 protein. (ii) Omission of the primary antibody also led to complete loss of labeling in all compartments.

The presence of Cpn10 in red blood cell or erythrocytes is surprising because these cells are devoid of mitochondria and various other organelles (Weiss, 1988). However, this result could be explained by assuming that although the mature

erythrocytes extrude all mitochondria, they retain specific mitochondrial proteins that may be required for carrying out particular functions. Our unpublished immunoelectron microscopy studies with Hsp60 antibodies indicate that similar to Cpn10, this protein is also present in the red blood cells. The functional role that these chaperone proteins play in the red blood cells is unclear at present, but based on their established role in protein folding and assembly of oligomeric protein complexes (Ellis and van der Vies, 1991), it is possible that these chaperones are involved in either the assembly or in the functional maintenance of hemoglobin molecules, which are the main constituents of red blood cells.

More surprising is our observation showing the presence of Cpn10 in various secretory granules (viz., zymogen granules in pancreatic acinar cells, PP granules in islet cells and growth hormone granules in anterior pituitary). The identity of the Cpn10 cross-reactive protein that is present in these granules is not yet established. However, based on the observation that Cpn10 has been shown to be identical to the early pregnancy factor present in the maternal blood, it is likely that the protein found in these granules is identical to Cpn10. In this regard it is of much interest that in our earlier work, Hsp60 has also been shown to be present in these granules (Cechetto et al., 2000). Thus, it is very likely that these proteins, in accordance with their chaperone function, are involved in the packaging of proteins in these granules and/or also in preventing the early activation of the proenzymes present in these granules (Ellis, 1987; Zeilstra-Ryalls et al., 1991; Cechetto et al., 2000). These granules may also provide the pathway for secretion of this protein into the blood stream, where it can serve other identified roles

such as early pregnancy growth factor (Quinn and Morton, 1992; Quinn et al., 1994) and immuno-suppressive function (Noonan et al., 1979; Morton, 1984; Morton, 1984).

The mechanism by which Cpn10 gets transported/ translocated to the various secretory granules is presently unknown. Since Cpn10 is encoded by a nuclear gene (Summers et al., 1996) and translated in cytoplasm, the protein found in these granules could either directly reach these granules via some unknown mechanism, or alternatively it could first enter mitochondria and then somehow gets translocated to these compartments. It is also possible that the Cpn10 found in these compartments is encoded for by a different gene (Summers et al., 1998; Fletcher et al., 2001) or by a differently spliced transcript. In the present instance, these possibilities cannot be easily distinguished. However, for the Hsp60 protein, which is also present in these granules and encoded by a single nuclear encoded gene (Venner et al., 1990), it has been previously established that the protein found in these extramitochondrial compartments is the mature form of the protein lacking the mitochondrial targeting presequence (Soltys and Gupta, 1999; Soltys and Gupta, 2000). Earlier work on this protein shows that the conversion of the precursor form of Hsp60 into its mature form does not occur nonspecifically in the cytoplasm but that it requires protein entry into mitochondria (Soltys and Gupta, 1996). These observations favor the possibility that the protein found in these granules is derived from the protein that was initially imported into mitochondria. Although the mechanism responsible for protein translocation from mitochondria to other compartments remains unknown at present, a number of possibilities have been discussed in our recent reviews (Soltys and Gupta, 1999; Soltys

and Gupta, 2000). In this context it is of interest to note that although the various secretory proteins found in these granules (viz. zymogen granules, growth hormone granules, PP granules) enter these compartments via the ER-Golgi pathway (Palade, 1975), results presented here for Cpn10, and described in our earlier work for Hsp60 (Soltys and Gupta, 1996), indicate that significant amounts of both these proteins are not found in the ER-Golgi compartments. In contrast, immuno-EM labeling studies with secretory proteins such as insulin or amylase show the expected labeling of the ER-Golgi pathway. These observations are suggestive that the proteins are reaching these compartments via a novel pathway. In an earlier report which also identified the presence of Cpn10 in zymogen granules, the Cpn10 reactivity was also seen along the ER-Golgi pathway (Vélez-Granell et al., 1994). However, this study employed a polyclonal antibody raised against the bacterial homolog from *Chromatium vinosum* (shows only about 34% identity to the rat protein), making the inference concerning the presence of this protein in the ER-Golgi pathway questionable (Cechetto et al., 2000). Lack of labeling for Cpn10 in the normal secretory pathway, namely the ER and Golgi could have also been due to one of many reasons, as discussed previously (Cechetto et al., 2000): (i) The protein might be complexed with other proteins while in transit through th ER and Golgi, thus masking the epitope(s). (ii) Levels of the protein in the ER and Golgi could be low and thus cannot be resolved by immunoelectron microscopy. (iii) There is a possibility that the ER and Golgi are subjected to enhanced fixation, leading to alteration in the epitope(s) of the protein.

The presence of Cpn10 at extramitochondrial sites, adds to the rapidly growing list of mitochondrial proteins that are present in a variety of extramitochondrial sites/compartments under normal physiological conditions and are involved in carrying out different functions. The other examples of such proteins include: (i) Hsp60 protein, which is involved in resistance to antimitotic drugs, immune response and cell signaling has also been shown to be present on cell surface (Ikawa and Weinberg, 1992; Jones et al., 1994; Soltys and Gupta, 2000), in peroxisomes (Soltys and Gupta, 1996), in insulin secretory granules (Brudzynski et al., 1992), growth hormone granules and zymogen granules (Cechetto et al., 2000). (ii) Mitochondrial Hsp70, which is responsible for the pulling of proteins into the matrix compartment (Hartl, 1996; Craig et al., 1993), has been localized at a variety of extramitochondrial sites and shown to be involved in antigen presentation and cellular senescence (Wadhwa et al., 1993a; VanBuskirk et al., 1989; Dahlseid et al., 1994). (iii) The mitochondrial aspartate aminotransferase has been shown to be identical to the plasma membrane fatty acid binding protein involved in the uptake of long chain free fatty acids in cells (Isola et al., 1995; Zhou et al., 1998; Bradbury and Berk, 2000). This protein has also been localized on cell surface in a variety of cells and tissues (Isola et al., 1995). (iv) The proteins P32 and TRAP-1 (tumor necrosis factor receptor associated protein 1), which are involved in a variety of extramitochondrial functions have both been shown to be primarily mitochondrial proteins that are also present on cell surface, nucleus and in various secretory granules (Soltys et al., 2000; Cechetto and Gupta, 2000). (v) Most interestingly in our recent work, cytochrome c, whose release from mitochondria is believed to play a central role in

the cascade of events leading to apoptotic cell death (Green and Reed, 1998; Kroemer et al., 1998), has also been shown to be present outside of mitochondria in a variety of compartments including pancreatic acinar lumen, zymogen granules, condensing vacuoles and in growth hormone granules, in large amounts, in normal tissues (Soltys et al., 2001). These results indicate that the translocation of cytochrome c from mitochondria to other specific sites also occurs under normal physiological conditions and its release in cytoplasm leading to apoptosis could be due to perturbation of the normal pathways.

It is currently believed that the primary function of mitochondria and mitochondrial resident proteins is related to oxidative phoshporylation and that under normal physiological conditions, these proteins do not exit mitochondria or play any functional roles outside of mitochondria (Poyton et al., 1992; Soltys and Gupta, 1999; Soltys and Gupta, 2000). In this context, our observation that a large number of well-characterized mitochondrial proteins, which were previously thought to reside and function exclusively in mitochondria, are present at a variety of specific locations in cells and involved in different functions is of great interest (Soltys and Gupta, 1999; Soltys and Gupta, 2000). Although the mechanisms responsible for the translocation of mitochondrial proteins to other destinations remain to be characterized, these results strongly indicate that the cellular functions of mitochondrial proteins are not restricted to within mitochondria and that mitochondria and mitochondrial-resident proteins play an important role in diverse cellular processes. In the past decade it has become clear that mutations affecting mitochondrial components are responsible for a broad spectrum of

genetic diseases affecting many different tissues and organs, often in a highly specific manner (Wallace, 1999; Schon, 2000). Based on the known function of mitochondria in oxidative phosphorylation, it has proven difficult to logically explain how mutations in different components of the respiratory chain, or mitochondrial protein synthesis machinery, could lead to clinically highly divergent phenotypes (Wallace, 1999; Schon, 2000). However, the involvement of mitochondrial resident proteins in a variety of extraneous functions should prove very useful in understanding the role of mitochondrial components in the etiology of different diseases.

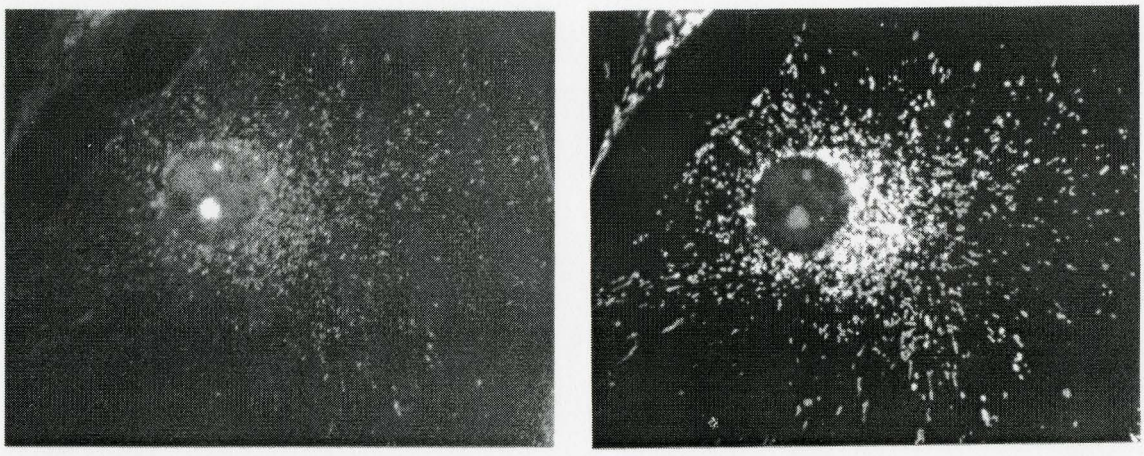

Figure 1: Double immunofluorescence labeling of cultured BS-C-1 cells with rabbit polyclonal antibody to Cpn10 (left panel) and a mouse monoclonal antibody to human Hsp60 (right panel).

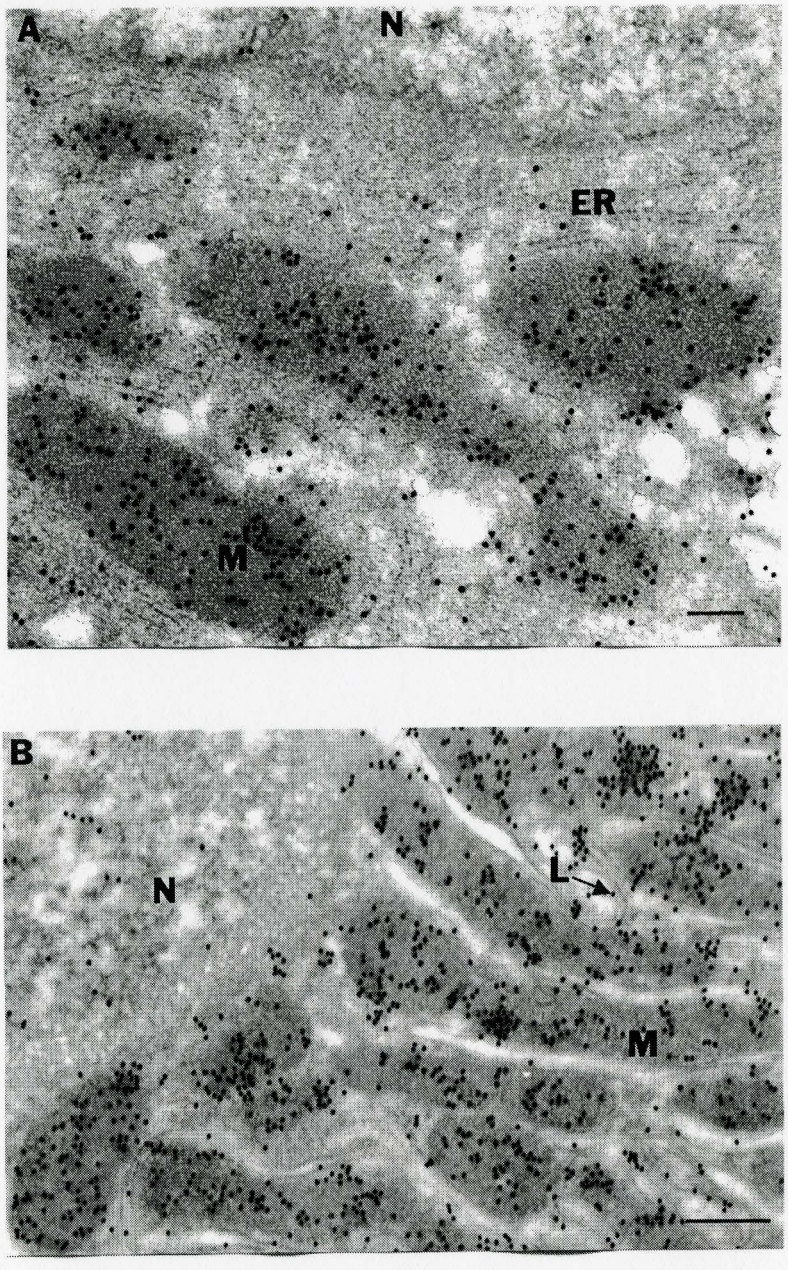

Figure 2: Immunogold localization of Cpn10 in rat liver and distal convoluted tubule region of the kidney. (A) Rat liver sections labeled with Cpn10. Cpn10 labeling is seen primarily within mitochondria. Bar = 200 nm. (B) In distal convoluted tubule region of kidney Cpn10 labeling was again primarily seen within the mitochondria. Detection of the primary antibody was carried out using 20 nm immuno-gold markers. Bar = 500 nm. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; L, lateral intercellular labyrinth.

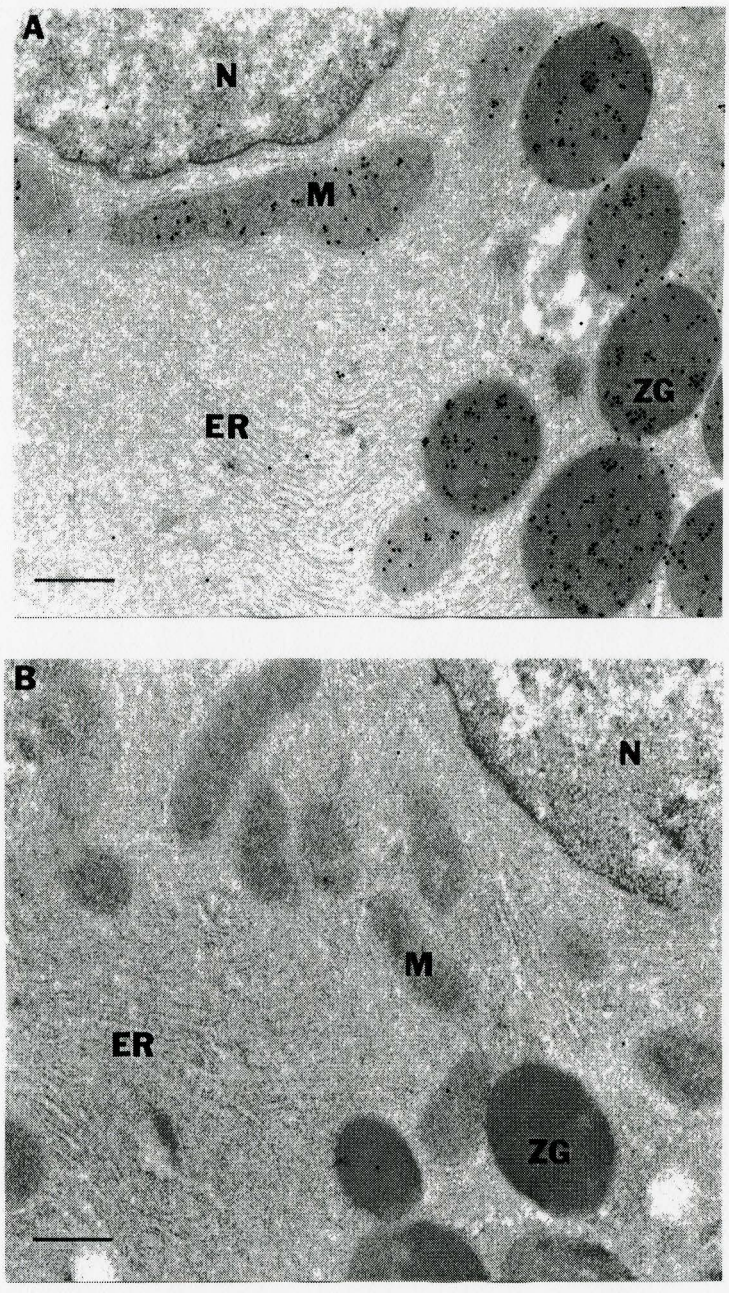

Figure 3: Subcellular localization of Cpn10 in rat pancreatic acinar tissue. (A) Rat pancreatic sections showing Cpn10 labeling in zymogen granules and mitochondria. Bar = 500 nm. (B) Same as in A, but the Cpn10 antibody was preadsorbed with purified recombinant human Cpn10 antigen before labeling. The secondary antibody was conjugated with 20 nm gold markers. Bar = 500 nm. N, nucleus; M, mitochondria; ZG, zymogen granules; ER, endoplasmic reticulum.

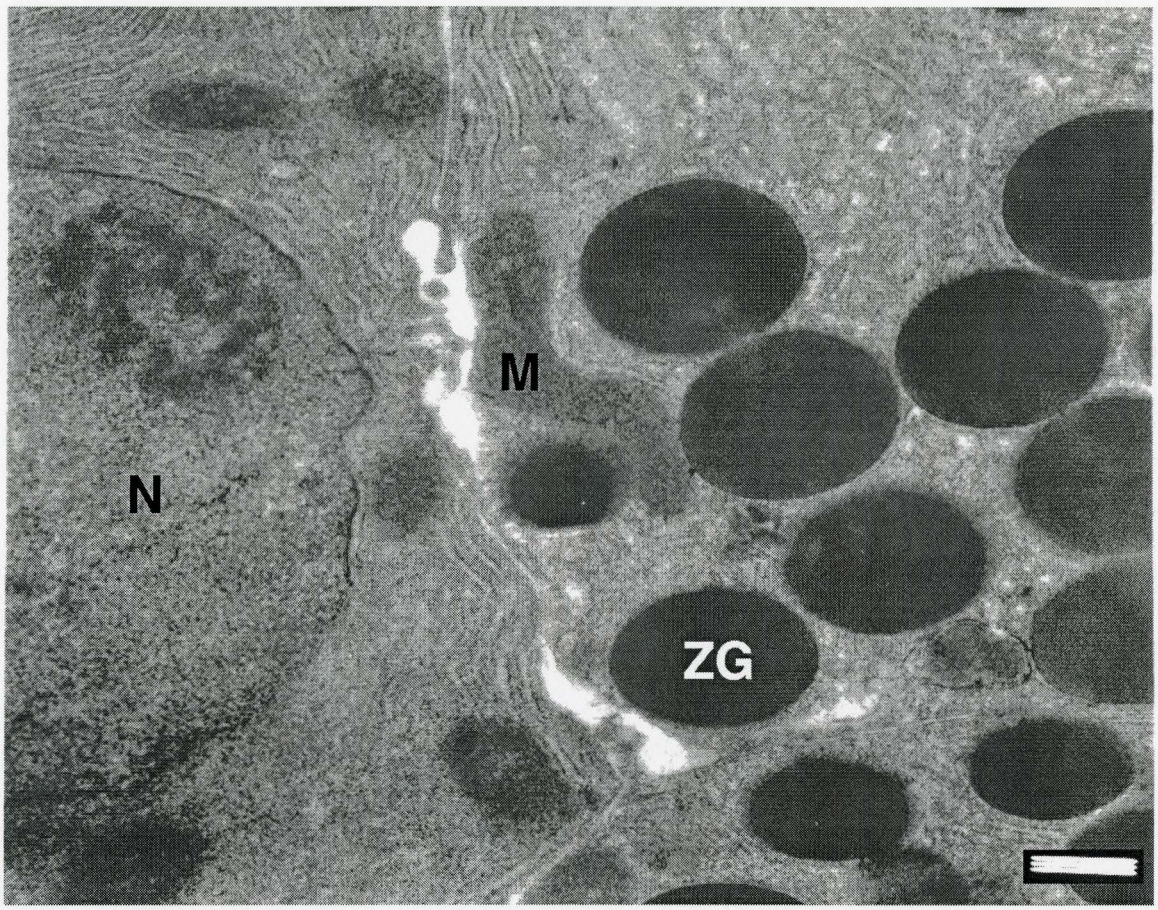

Figure 4: Labeling in LR Gold section of rat pancreas was absent when primary antibody was omitted. No labeling was observed in zymogen granules or mitochondria. Goat anti-rabbit secondary antibody bound to 20 nm gold particles were used. Bar = 500 nm. M, mitochondria; N, nucleus; ZG, zymogen granule.

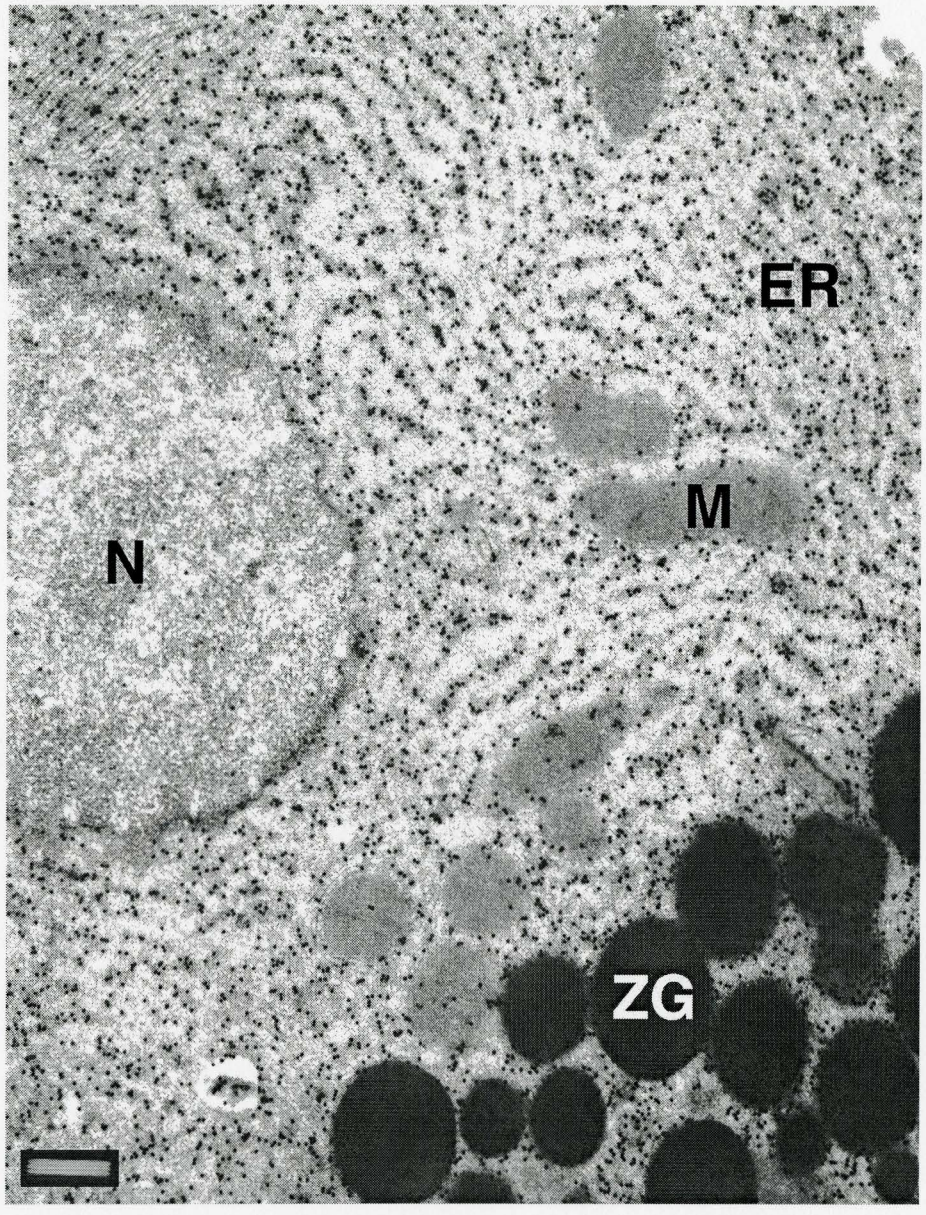

Figure 5: Subcellular localization of α-amylase in LR Gold section of rat pancreatic acinar tissue. Labeling of α-amylase is seen all along the endoplasmic reticulum and in zymogen granules. No labeling is seen in mitochondria. Detection of the primary antibody was carried out using 20 nm immuno-gold markers. Bar = 500 nm. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; ZG, zymogen granule.

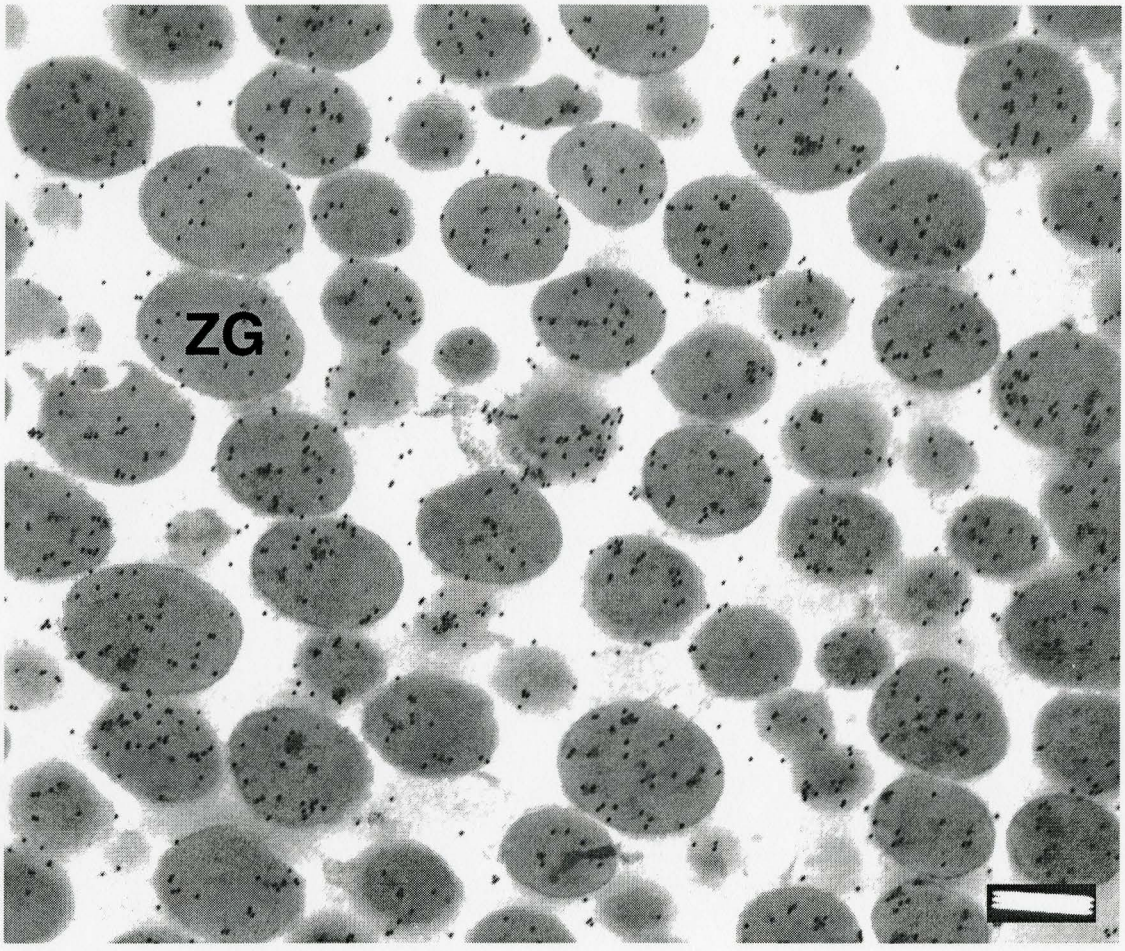

Figure 6: Cpn10 labeling in purified bovine zymogen granules. Cpn10 labeling is seen within electron dense zymogen granules. Bar = 500 nm. ZG, zymogen granules.

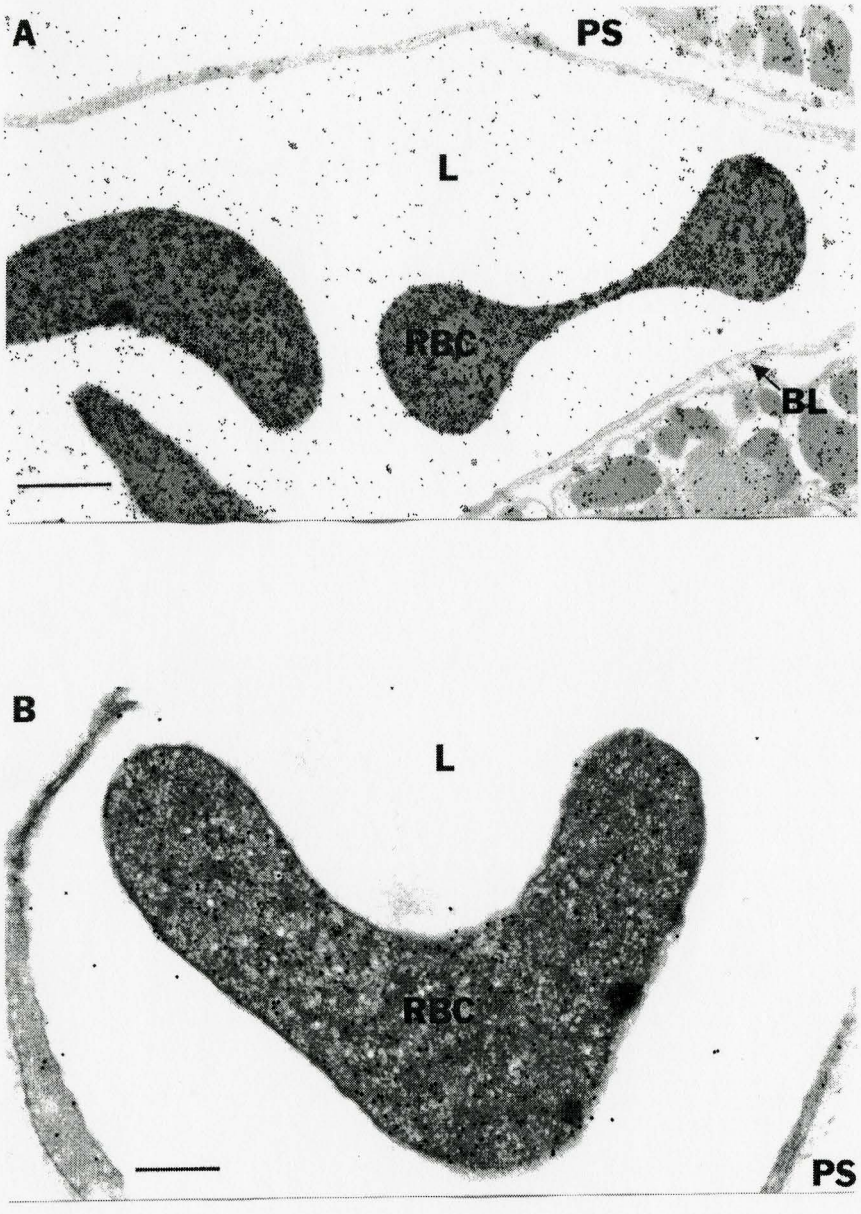

Figure 7: Presence of Cpn10 in red blood cells found within a blood vessel in (A) rat heart (Bar = 1 μm) and (B) pancreas (Bar = 500 nm). Reactivity is seen mainly in red blood cells with labeling in other compartments at background levels. RBC, red blood cell; PS, pericapillary space; L, lumen; BL, basal lamina.

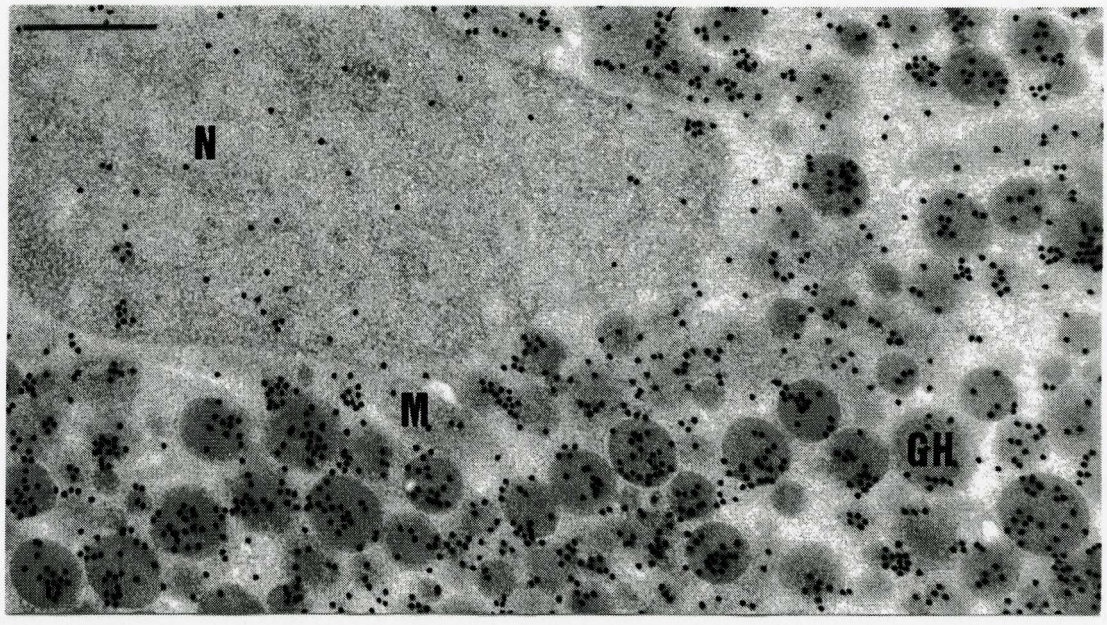

Figure 8: Subcellular localization of Cpn10 in rat anterior pituitary sections. Cpn10 localization is seen within mitochondria and growth hormone granules. Bar = 500 nm. N, nucleus; M, mitochondria; GH, growth hormone granules;

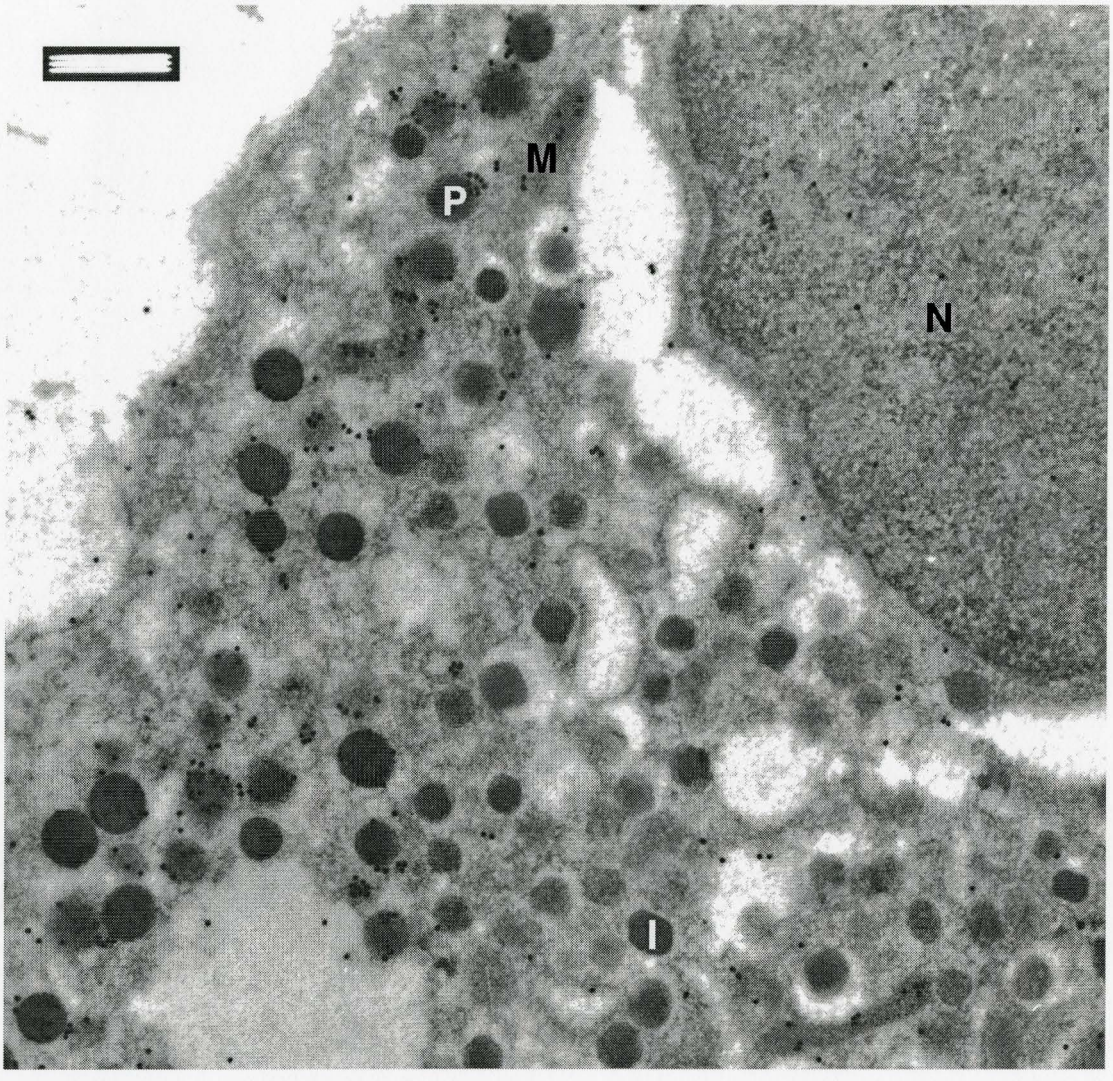

Figure 9: Subcellular localization of Cpn10 in pancreatic islet. Cpn10 labeling was selectively seen in PP cell secretory vesicles, mitochondria and not in insulin secretory granules. Bar = 500 nm. M, mitochondria; N, nucleus; P, PP cell secretory granules; I, insulin secretory granules.

Chapter 4.0:

Immunogold Localization of Mitochondrial Aspartate Aminotransferase in Mitochondria and on the Cell Surface in Normal Rat Tissues

4.1 Abstract

Mitochondrial aspartate aminotransferase (mAspAT) (E.C. 2.6.1.1), an important enzyme in amino acid metabolism, is identical to a fatty acid binding protein (FABPpm) isolated from plasma membranes of several cell types. Employing a monospecific polyclonal antibody to rat mAspAT, we have used immunogold electron and immunofluorescence microscopy to study the subcellular distribution of mAspAT in various mammalian tissues and BS-C-1 monkey kidney cells respectively. Immunofluorescent labeling of BS-C-1 cells with this antibody, showed predominant labeling of only mitochondria. Immunogold labeling of rat tissue sections embedded in LR Gold resin showed strong labeling of mitochondria in all tissues (viz. liver, pancreas, pituitary, spleen, heart , kidney, submandibular gland). In addition, strong and specific labeling was also observed at a number of non-mitochondrial sites including various locations in kidney such as, on cell surface in distal tubules and cortical collecting ducts, in condensing vacuoles, along cell boundaries between adjoining cells, and in endothelial cells lining capillaries in the glomerulus. Surface labeling due to mAspAT was also seen in arteriolar endothelial cells and in lymphocytes. These findings support the previous identification of mAspAT as both a mitochondrial enzyme and a plasma membrane protein. It is suggested that in accordance with its established role in other cells and tissues, the surface located mAspAT in kidney and endothelial cells is involved in the fatty acid transport process. The dual-localization of mAspAT, as well as a large number of other mitochondrial proteins (viz. Hsp60, Hsp10, Cytochorome c, TRAP-1 and P32) in recent studies, within both mitochondria and at various specific extramitochondrial sites

raises fundamental questions about the role of mitochondria in cell structure and function, and about the mechanisms that exist in normal cells for protein translocation from mitochondria to other compartments. These observations are also relevant for understanding the role of mitochondria in different diseases.

4.2 Introduction

Mitochondrial aspartate aminotransferase (mAspAT) (E.C. 2.6.1.1) is a well studied enzyme that plays a major role in amino acid metabolism and provides an important route for the import of reducing equivalents into mitochondria through its participation in the malate:aspartate shuttle (Christen and Metzler, 1985). In their studies initially unrelated to mAspAT, Berk et al. previously identified a 43 kDa plasma membrane fatty acid binding protein (FABPpm) in a number of different tissues, which was shown to be responsible for the facilitated uptake of long chain free fatty acid (FFA) in cells (Stremmel et al., 1985a; Stremmel et al., 1986; Schwieterman et al., 1988). Structural analysis of highly purified FABPpm from rat liver plasma membrane, led to the unexpected discovery that FABPpm was very similar to mAspAT (Berk et al., 1990). Subsequent studies demonstrated that the two proteins were both immunologically and functionally identical (Stump et al., 1993). For example, in vivo transfection of NIH 3T3 cells with mAspAT cDNA led to de novo expression on the cell surface of a 43 kDa protein immunoreactive with antibodies to both mAspAT and FABPpm (Isola et al., 1995). Moreover, after transfection, these cells, normally deficient in FFA uptake, exhibited saturable uptake of FFA that was selectively inhibitable by antibodies to mAspAT (Isola et al., 1995). Further evidence of a role for mAspAT in FFA uptake derives from studies in HuH7 hepatoblastoma cells. Culture of these cells in ethanol, induced a significant increase in the expression of mAspAT mRNA, which was highly correlated with an increase both in the amount of mAspAT protein on the cell surface and increased facilitated uptake of FFA (Zhou et al., 1998). Thus, mAspAT appears to be a

member of a growing group of mitochondrial proteins that have different functions in different cellular compartments (Smalheiser, 1996; Jeffery, 1999; Soltys and Gupta, 1999; Soltys and Gupta, 2000).

In the past, work on the sub-cellular distribution of mAspAT has focused almost exclusively on the liver, and the extent to which mAspAT is present at extramitochondrial locations in other tissues is not known. Since mAspAT is a highly conserved protein, with extensive immunological cross-reactivity among mammalian species (Christen and Metzler, 1985; Winefield et al., 1995), in the present report, we have used a highly specific polyclonal antibody to rat liver mAspAT to examine the subcellular distribution of mAspAT in various rat tissues using immunoelectron microscopy. Our results confirm the inference that mAspAT is primarily a mitochondrial protein that is abundantly present in mitochondria of all cells and tissues examined. However, our labeling studies also confirm and provide strong evidence that significant quantities of mAspAT are also present on the plasma membrane of several, but not all, of the tissues examined. These results support the concept that while mAspAT is primarily a mitochondrial protein, it is also present and functions at extramitochondrial sites, as has recently been reported for several other mitochondrial proteins (Soltys and Gupta, 1996; Soltys and Gupta, 1999; Cechetto et al., 2000; Cechetto and Gupta, 2000; Soltys et al., 2000; Soltys and Gupta, 2000; Soltys et al., 2001).

4.3 Results

The antibody to mAspAT used in the present work has been characterized extensively (Stremmel et al., 1986; Berk et al., 1990; Stump et al., 1993; Isola et al., 1995). In Western blots of tissue extracts, plasma membrane preparation, as well as in purified rat liver mitochondrial fractions (unpublished results), it reacts with a single band of 43-kD, corresponding to mAspAT (Stremmel et al., 1986; Berk et al., 1990; Stump et al., 1993; Isola et al., 1995; Zhou et al., 1998).

Subcellular Localization of mAspAT in BS-C-1 Cells

Immunofluorescence studies with this antibody in permeabilized mouse 3T3 cells show specific labeling of mitochondria (Isola et al., 1995). However, in non-permeabilized 3T3 cells, particularly those which overexpress the cDNA for mAspAT, strong and specific labeling on cell surface was also observed (Isola et al., 1995).

This antibody to mAspAT and a mouse monoclonal antibody to Hsp60 were employed for double immunofluorescence labeling of cultured African green monkey, BS-C-1 cells. The observed punctate and bead-string shaped labeling pattern for mAspAT (Figure 1, left panel), is typical of mitochondrial staining. Labeling pattern observed with Hsp60 antibody (Figure 1, right panel), a mitochondrial protein is identical to that observed with mAspAT antibody, thus demonstrating that mAspAT antibodies are labeling mitochondria. There is no indication of any cell surface or extramitochondrial labeling from this experiment.

Subcellular Localization of mAspAT in Normal Rat Tissues

Subcellular localization of mAspAT in rat tissue sections, prepared as described in Materials and Methods, was carried out using immunogold electron microscopy. Tissue sections embedded in LR Gold were probed with polyclonal antibodies to mAspAT, followed by detection with secondary antibodies conjugated to 20 nm gold particles. The specificity of the secondary antibodies used in this study was confirmed by carrying out the labeling of tissue sections without the primary antibody, which consistently resulted in abolishment of labeling. This provides evidence that the secondary antibodies bind specifically to the primary antibody and do not react with the acrylic resin in which the tissue is embedded. The results of our studies with different tissue sections are as follows:

Liver: Subcellular localization of mAspAT in a representative rat liver LR Gold tissue section is shown in Figure 2. As seen, the antibody showed strong labeling of mitochondria with very little labeling present in any of the other compartments such as, nucleus, ER or peroxisomes. The lack of labeling in peroxisomes is of interest because in our earlier work, antibodies to another mitochondrial protein, Hsp60, have been shown to label these structures (Soltys and Gupta, 1996; Cechetto et al., 2000). The identity of these structures as peroxisomes was confirmed by labeling with antibody to catalase, which selectively labeled peroxisomes but showed no labeling of mitochondria (not shown).

Pancreas: Figures 3A shows the results of labeling in rat pancreatic acinar cells. In pancreatic acinar tissue sections strong labeling was observed within mitochondria and the labeling in other compartments such as, nucleus, ER and or zymogen granules (ZG)

was very weak and at background levels. The lack of mAspAT labeling in zymogen granules was further confirmed by western immunoblotting (Figure 3B). Rabbit polyclonal antibody to mAspAT shows specific immunoreactivity to a single protein of about 43 kDa in bovine mitochondrial fraction. This corresponds to the expected size of mature mAspAT. There is no immunoreactivity seen in the purified bovine zymogen granules fraction, thus indicating lack of mAspAT in these granules. Furthermore, as visualized by immunoelectron microscopy, mAspAT did not show any labeling in purified bovine zymogen granules (not shown). The lack of reactivity of mAspAT antibody in zymogen granules is of particular interest because antibodies to a variety of other mitochondrial proteins that we have previously examined (viz. Hsp60 (Cechetto et al., 2000), TRAP-1 (Cechetto and Gupta, 2000) and P32 protein (Soltys et al., 2000), cytochrome c (Soltys et al., 2001)), all showed significant reactivity in these structures. The identity of ZGs was verified by labeling pancreatic acinar tissue sections with an antibody to amylase, which selectively labeled zymogen granules and endoplasmic reticulum. Figure 4, shows the results of labeling in insulin secretory cells. In pancreatic insulin secretory cells labeling of mAspAT was seen mainly in mitochondria. Labeling was not seen in insulin secretory granules and the labeling seen in the cytoplasm and nucleus was at background levels.

Pituitary and submandibular glands: Mitochondrial labeling was also seen in anterior pituitary (Figure 5) and serous cells found in the submandibular gland. In the anterior pituitary, labeling was specific for mitochondria, with no labeling seen in growth hormone granules, where antibodies to a number of other mitochondrial proteins

have been shown to localize (Soltys et al., 2001; Soltys et al., 2000; Cechetto et al., 2000; Cechetto and Gupta, 2000). Likewise, in serous cells found in the submandibular gland, labeling was again specific for mitochondria with hardly any labeling seen in secretory granules (not shown). The labeling in the cytoplasm and nucleus in both these tissues was negligible and at background levels.

Kidney: In contrast to the tissues described thus far, mAspAT was localized to a variety of extramitochondrial locations in different regions of the rat kidney. In the distal convoluted tubules, mAspAT reactivity was found both in mitochondria and in dense structures that have been identified as condensing vacuoles (Figure 6A). The condensing vacuoles arise from the endocytotic apparatus and their contents eventually end up in lysosomes (Bulger, 1988). No labeling was seen in any other compartments. In the ascending part of the thick distal tubule of rat kidney, both specific mitochondrial labeling and labeling of the basal region of the cell surface were observed (Figure 6B). The labeling on the basal cell surface is not homogenous, and seems to be concentrated in regions with protrusions.

Surface labeling for mAspAT was also seen in other regions of the kidney, such as cell boundaries of adjoining kidney cells (Figure 7A). The identity of cells showing such labeling is presently unclear. Specific labeling was also observed in mitochondria, and no significant labeling is seen in the cytosol or nucleus of these cells. Figure 7B, shows the specific labeling of mAspAT along the luminal side of endothelial cells lining capillaries of rat kidney glomerulus. Although no blood cells were present within the capillaries they were identified as capillaries based on the orientation of the pedicels.

Reactivity for mAspAT in kidney cortical collecting duct tissue sections was found exclusively in mitochondria and on the apical cell surface (Figure 8). It is to be noted that the labeling seen on cell surface is not uniform but generally restricted to cell surface protrusions along the microvilli. In contrast to the distal convoluted region, in the proximal convoluted tubule region of rat kidney, labeling was seen only in mitochondria with no surface labeling observed in the brush border region (not shown).

Other sites: Surface localization of mAspAT was also seen in endothelial cells lining arterioles (Figure 9A). The labeling seen on the surface in this case was again not uniform, but concentrated in the projections present on the cell surface. It is of note that similar labeling has also been observed for TRAP-1 (Cechetto and Gupta, 2000) and P32 (Soltys et al., 2000) proteins, both of which are mitochondrial proteins. mAspAT reactivity was also seen on the cell surface in lymphocytes (Figure 9B). The lymphocyte surface labeling is also not uniform and resembles that on the endothelial cell surface. No appreciable labeling is seen in any other compartment in lymphocytes. In a number of other tissues studied, namely, spleen and heart, prominent labeling was again seen for mitochondria with no other sites with specific labeling identified (not shown).

4.4 Discussion

mAspAT has previously been identified as both a mitochondrial enzyme and, in a limited number of cell types, as a plasma membrane fatty acid binding protein. Although the presence of mAspAT on plasma membranes has been investigated previously by immunofluorescence and Western blotting in several tissues and in cell lines (Stremmel et al., 1985b; Stremmel et al., 1986; Berk et al., 1990; Stump et al., 1993; Isola et al., 1995; Zhou et al., 1998), and by immunoelectron microscopy in a rat hepatoblastoma cell line (Zhou et al., 1998), the current study represents the first detailed application of immunoelectron microscopic methods to examine the subcellular localization of mAspAT in multiple tissues. Our survey of different tissues for mAspAT localization shows that although mAspAT is abundantly found in mitochondria in all cases, significant and specific reactivity to mAspAT antibody was also found at the cell surface in a number of tissues. While the presence of mAspAT to mitochondria was expected in view of extensive earlier work identifying this organelle as the principal site of its enzymatic functions (Christen and Metzler, 1985), the localization of mAspAT on the cell surfaces in various regions of the kidney and in arteriolar endothelial cells represents a novel finding that is of considerable significance. The surface labeling due to mAspAT, was not found in all tissues, but restricted to only certain tissues, and even within each tissue reactivity was seen only in certain specialized cells, as exemplified by the findings in rat kidney. This suggests that mAspAT is specifically targeted to the cell surface only in certain cells or tissues, to meet specific functional needs. These observations raise two obvious questions: (i) What is the trafficking pathway by which

mAspAT reaches the plasma membrane? and (ii) What is the functional significance of its presence there?

With regard to the first of these questions, it is well established that mAspAT is encoded by nuclear DNA, and is translated on free cytosolic ribosomes as a larger pre-protein containing a N-terminal mitochondrial targeting sequence, which is not present in the mature protein (Sonderegger et al., 1982). Since the targeting sequence is encoded by exon 1 of the mAspAT gene, with the exon 1:exon 2 splice site occurring at the first codon of the mature protein (Joh et al., 1985; Lain et al., 1998), it was originally considered that the distribution of the protein to extramitochondrial sites might be the consequence of alternative RNA splicing, creating several mAspAT mRNAs having different targeting sequences. However, subsequent studies have established that there is only a single message for mAspAT in cells in which there is abundant mAspAT on the plasma membrane, indicating that the distribution of mAspAT protein to different sub-cellular sites does not reflect alternative RNA splicing and creation of multiple messages (Bradbury and Berk, 2000). mAspAT isolated from plasma membranes of hepatocytes, adipocytes, and cardiac myocytes has been shown to be the mature form of the protein lacking the N-terminal targeting presequence (Stremmel et al., 1985a; Schwieterman et al., 1988; Berk et al., 1990; Stump et al., 1993). In our earlier work with Hsp60 protein, which is also found at a variety of extramitochondrial sites, evidence has been presented that the conversion of the precursor form of the protein to its mature form found at other sites, does not occur if the mitochondrial import of the preprotein is inhibited (Soltys and Gupta, 1996). These results favor the possibility that the proteins are first imported into

mitochondria and, after cleavage of the pre-sequence, they are translocated to the other sites (Soltys and Gupta, 1999; Soltys and Gupta, 2000). The mechanism(s) by which proteins are translocated from mitochondria to other sites is presently unclear, however a number of possibilities have been discussed (Soltys and Gupta, 2000).

There is now extensive evidence supporting the view that the mAspAT on the plasma membrane is central to the process of facilitated FFA uptake and efflux (Stremmel et al., 1985a; Stremmel et al., 1986; Schwieterman et al., 1988; Berk et al., 1990; Stump et al., 1993; Isola et al., 1995; Zhou et al., 1998; Berk and Stump, 1999), in many different cells and tissues, of which isolated hepatocytes and hepatoblastoma cell lines have been most extensively studied. In this context, our observation that the antibodies to mAspAT, show strong and specific labeling of cell surface in various regions of the kidney, is of much interest. The different regions of kidney where mAspAT is specifically localized include, the basal cell surface in distal convoluted tubule region, in condensing vacuoles which are a part of the endocytotic apparatus on the apical cell surface (Bulger, 1988), microvilli of cortical collecting duct cells, and along the cell boundaries of adjoining cells in an unidentified region of the kidney. In contrast, no mAspAT reactivity was observed at either the apical or basal cell surface of the proximal convoluted tubule. Although the renal uptake or disposition of FFA has not been extensively studied, in part because these tightly albumin-bound compounds normally experience little or no glomerular filteration, our observation that mAspAT is present in appreciable amounts in these specific kidney regions, provides strong

suggestive evidence that these regions of kidney are actively engaged in the uptake and/or efflux of FFA.

In addition to the different regions in kidney, specific cell surface labeling was also observed in endothelial cells lining blood vessels and in lymphocytes. The surface labeling in these cells, as also in the case of kidney, was not uniform but localized to specific regions, often in the form of membrane projections which could comprise of vesicles that are either fusing with or budding from the membrane. The need for specific transport mechanisms in endothelial cells to move FFA across the endothelial barrier from the vascular lumen to the extravascular, extracellular space has been speculated upon (Berk and Stump, 1999; Vyska et al., 1991) and our results provide suggestive evidence for the existence of such a process in these cells. Thus, the various sites on the plasma membrane where mAspAT is seen may have in common the shared characteristics of facilitated FFA transport processes. Conversely, neither the existence of nor an apparent need for facilitated FFA transport is evident in acinar or insulin secretory cells of the pancreas, the anterior pituitary or submandibular gland, sites at which no mAspAT was detected on the plasma membrane.

Our observation that mAspAT, which is primarily a mitochondrial protein, is also found on cell surface in many different cells and tissues, where it is apparently involved in FFA transport, raises fundamental questions concerning the cellular functions of mitochondrial proteins. In recent years, many other examples have been reported of proteins that, like FABPpm, were initially identified on the basis of non-mitochondrial functions, but were subsequently identified as mitochondrial proteins (Soltys and Gupta,

1999; Soltys and Gupta, 2000). These proteins include: the mitochondrial heat shock protein Hsp60, which has been implicated in resistance to antimitotic drugs (Gupta, 1990; Picketts et al., 1989), amino acid transport (Jones et al., 1994), signal transduction (Ikawa and Weinberg, 1992; Khan et al., 1998), and immune response (Kaur et al., 1993; Khan et al., 1998); the mitochondrial heat shock protein Hsp70, which has been implicated in cellular senescence (Wadhwa et al., 1993b) and antigen presentation (Domanico et al., 1993); the Hsp60 co-chaperone cpn10, which has been shown to be identical to the early pregnancy growth factor found in maternal blood (Cavanagh, 1996); the tumor necrosis factor receptor associated protein TRAP-1 (Cechetto and Gupta, 2000; Felts et al., 2000), which has been shown to interact with the type I tumor necrosis factor and the retinoblastoma protein (Chen et al., 1996); the β-subunit of mitochondrial F1-ATP synthase, reported to occur on the basolateral domain of the hepatocyte plasma membrane and to function as an organic anion binding protein (Wolkoff and Chung, 1980; Goeser et al., 1990); the protein P32, involved in nuclear functions as well as acting at the cell surface as a receptor for complement C1q (Muta et al., 1997; Soltys et al., 2000); and a growing number of others. The work on these and other proteins have been recently reviewed by us (Soltys and Gupta, 1999; Soltys and Gupta, 2000). Most interestingly our recent work shows that, cytochrome c, whose release from mitochondria is believed to play a central role in the cascade of events leading to apoptotic cell death (Soltys et al., 2001), is also present outside of mitochondria in a variety of compartments including pancreatic zymogen granules and condensing vacuoles as well as in growth hormone granules, in normal tissues (Soltys et al., 2001). These results indicate that the

translocation of cytochrome c from mitochondria to other specific sites also occurs under normal physiological conditions and its release in cytoplasm leading to apoptosis could be due to perturbation of the normal pathways.

It is currently believed that the primary function of mitochondria and mitochondrial resident proteins is related to oxidative phoshporylation and that under normal physiological conditions these proteins do not exit mitochondria or play any functional roles outside of mitochondria (Scheffler, 1999). In this context, our observation that a large number of well-characterized mitochondrial proteins, including mAspAT, which were previously thought to reside and function exclusively in mitochondria, are present at a variety of specific locations in cells and involved in different functions have led us to postulate the existence of specific mechanism(s) by which certain proteins could exit mitochondria, to assume additional functions at specific extramitochondrial sites (Soltys and Gupta, 1999; Soltys and Gupta, 2000). Although such mechanisms remains to be characterized, these observations strongly indicate that the cellular functions of mitochondrial proteins are not restricted to within mitochondria and that mitochondria and mitochondrial-resident proteins play an important role in diverse cellular processes (Soltys and Gupta, 1999; Soltys and Gupta, 2000). In recent years it has become clear that mutations affecting mitochondrial components are responsible for a broad spectrum of genetic diseases affecting many different tissues and organs, often in a highly specific manner (Wallace, 1999; Schon, 2000). Based on the known function of mitochondria in oxidative phosphorylation, it has proven difficult to logically explain these observations (Schon, 2000). However, our observation that

mitochondrial proteins are involved in a variety of extraneous functions, provides potentially useful insight that could prove helpful in understanding the role of mitochondria in the etiology of different diseases.

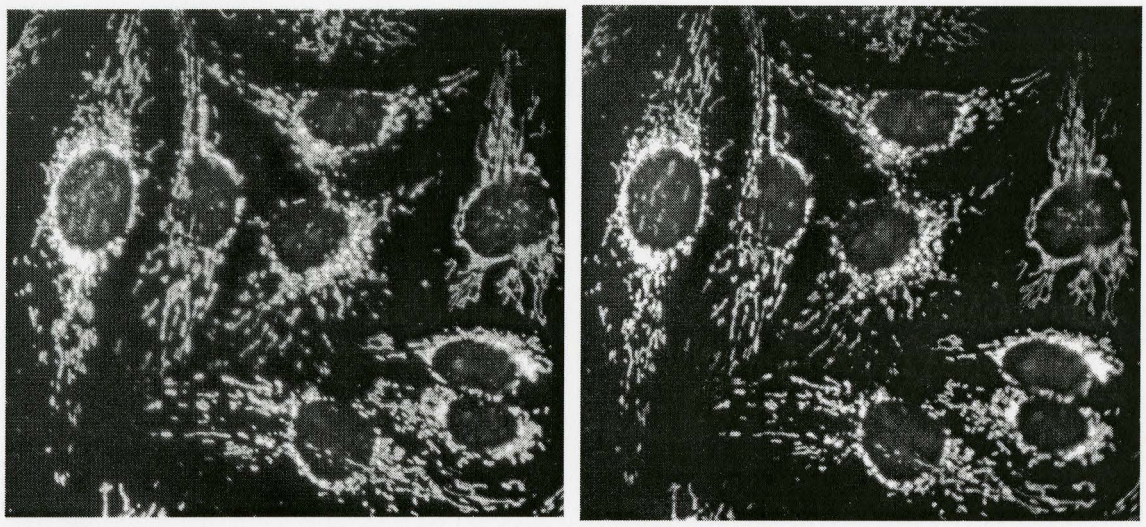

Figure 1: Double immunofluorescence labeling of cultured BS-C-1 cells with a rabbit polyclonal antibody to mAspAT (left panel) and a mouse monoclonal antibody to human Hsp60 (right panel).

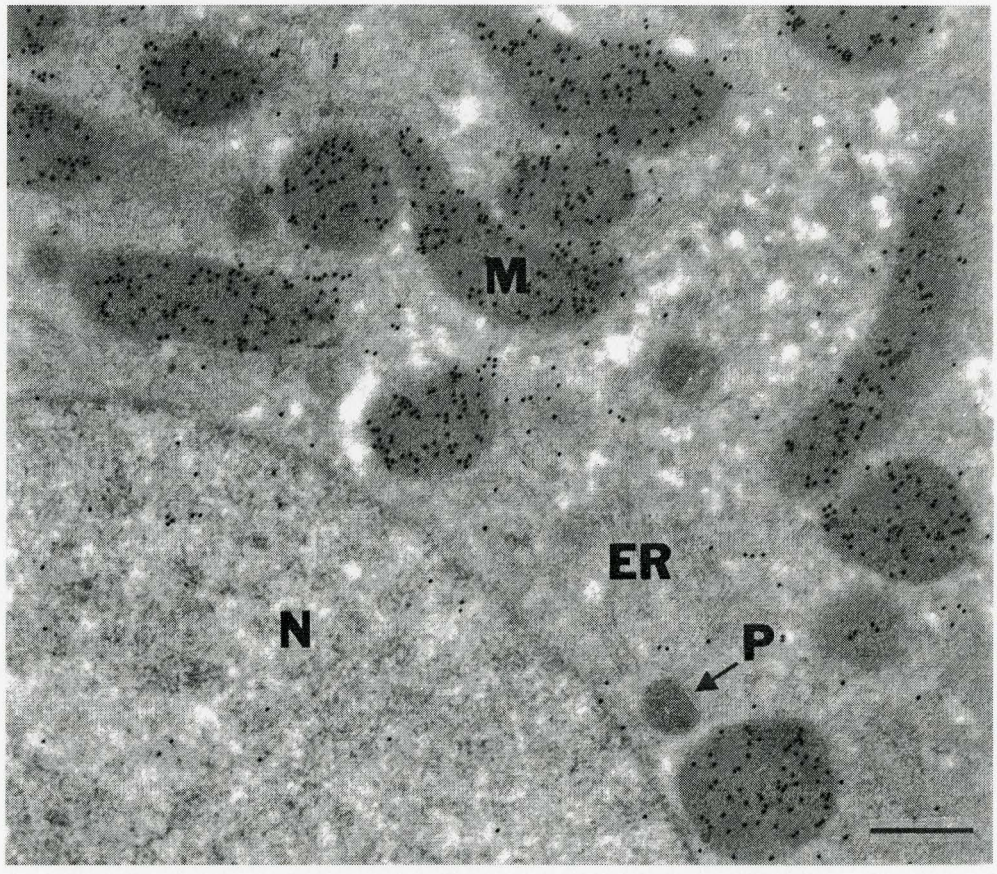

Figure 2: Subcellular localization of mAspAT in rat liver LR Gold sections using immunogold electron microscopy. Labeling was carried out using a polyclonal antibody to mAspAT and then followed by secondary antibody bound to 20 nm gold particles. Labeling in this section is seen exclusively in the mitochondria, with no labeling present in peroxisomes. Bar = 500 nm. N, nucleus; M, mitochondria; P, peroxisomes; ER, endoplasmic reticulum.

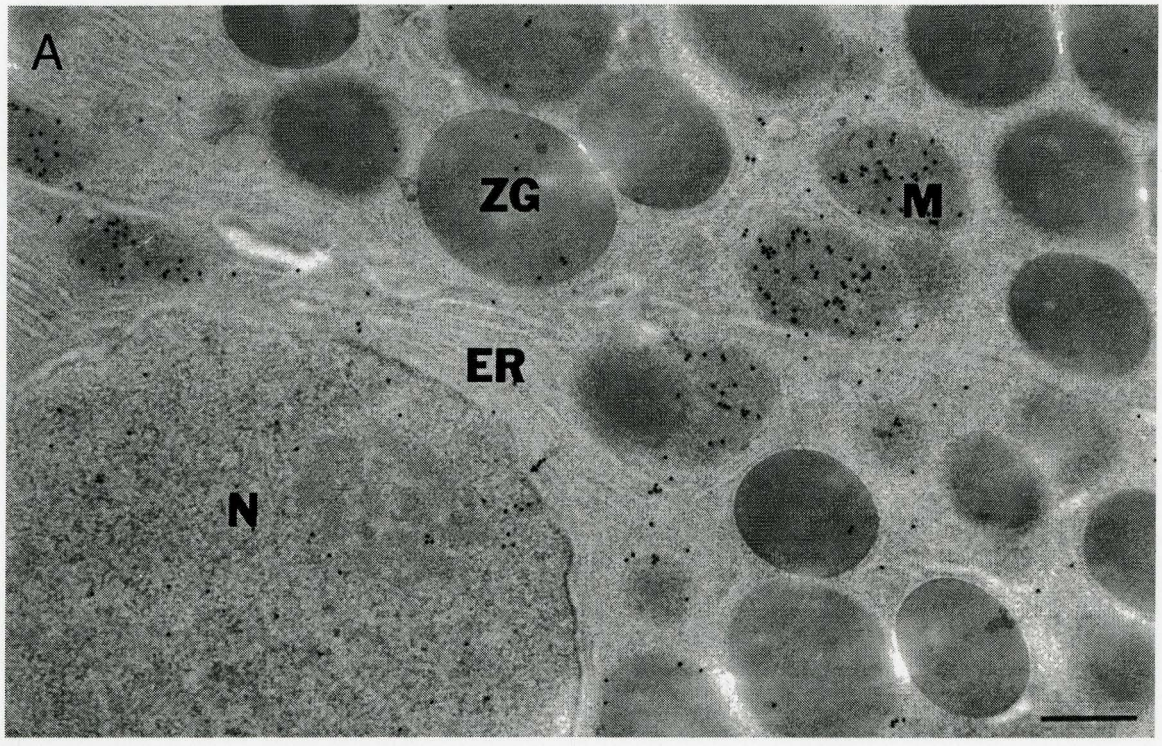

Figure 3A: Subcellular localization of mAspAT in rat pancreatic acinar tissue, using immunogold electron microscopy. Reactivity in pancreatic acinar tissue LR Gold sections is seen almost exclusively in mitochondria; labeling seen in all other compartments is at background levels. Bar = 500 nm. 20 nm gold particles. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; ZG, zymogen granules.

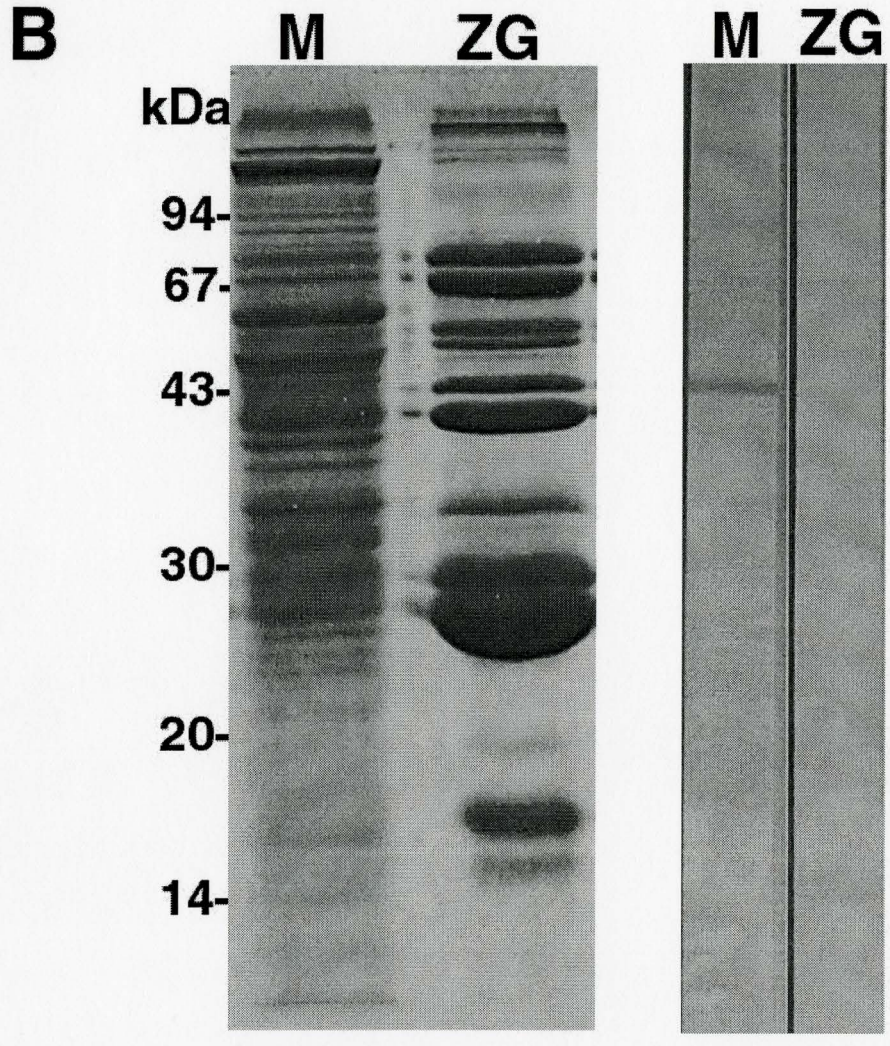

Figure 3B: SDS-PAGE and western blot analysis of purified bovine zymogen granules and mitochondria. Left panel: SDS-PAGE of purified zymogen granules and mitochondrial proteins. Proteins were visualized by staining the gel with Coomassie blue. Right panel: Western immunoblot analysis of purified bovine pancreatic zymogen granules and mitochondria using a rabbit polyclonal antibody to mAspAT. The bovine mitochondrial lane shows an immunoreactive band corresponding to 43 kDa. There are no reactive bands in the purified zymogen granule lane. M, mitochondria; ZG, zymogen granules.

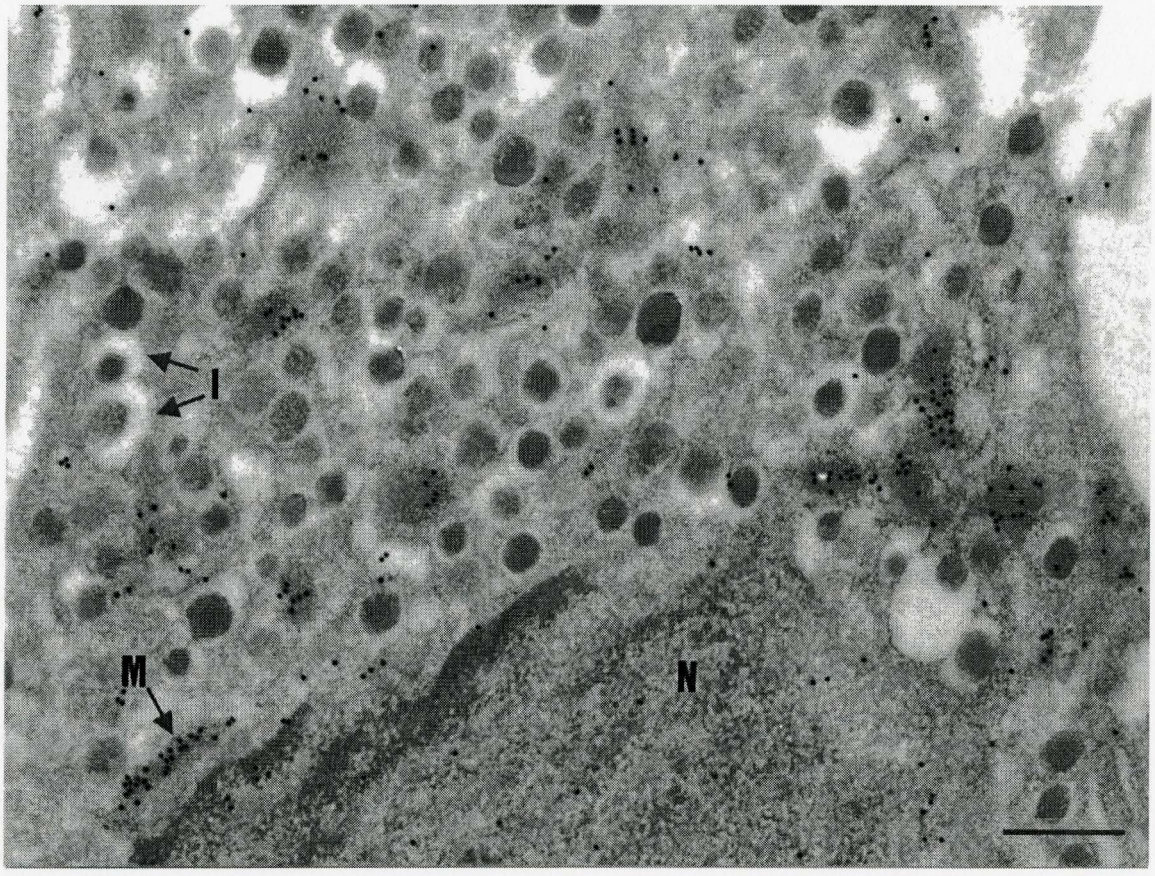

Figure 4: Subcellular localization of mAspAT in rat pancreatic islet. Localization of mAspAT in insulin secretory cells is within mitochondria. No reactivity is seen in the insulin secretory granules. Bar = 500 nm. 20 nm gold particles. N, nucleus; M, mitochondria; I, insulin secretory granules.

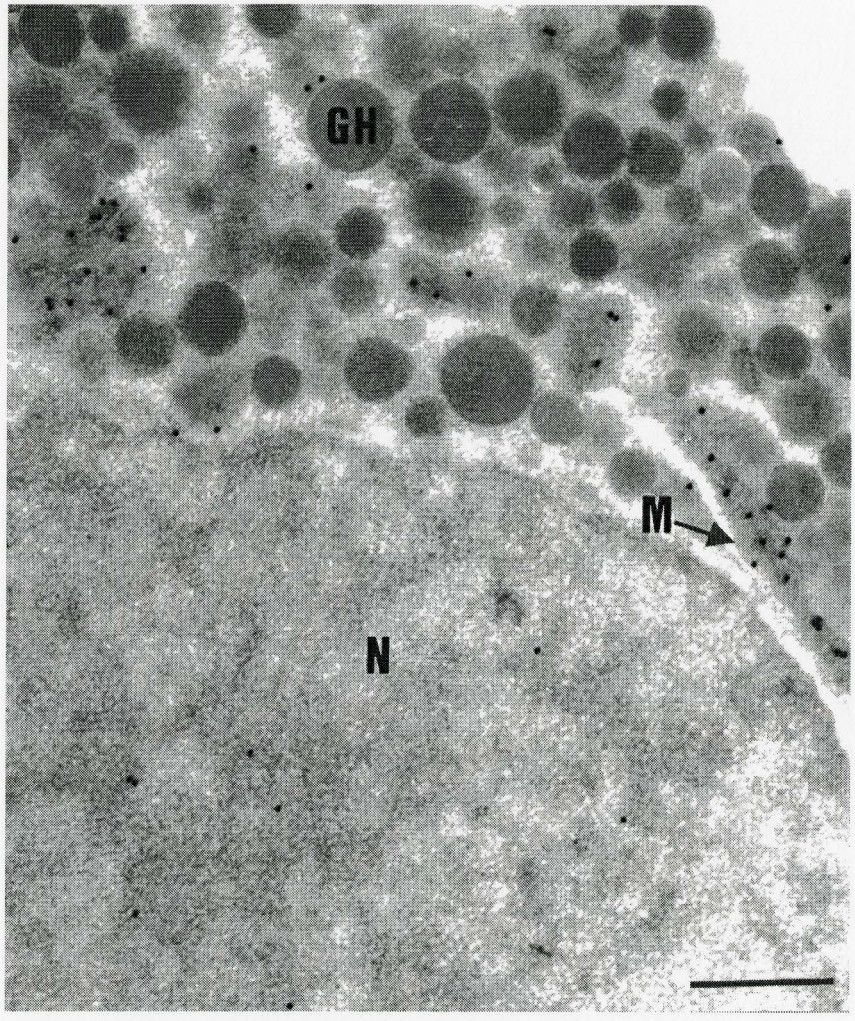

Figure 5: Mitochondrial reactivity for mAspAT is seen in rat anterior pituitary. There is no labeling seen in the growth hormone granules. Bar = 500 nm. 20 nm gold particles. N, nucleus; M, mitochondria; GH, growth hormone granules.

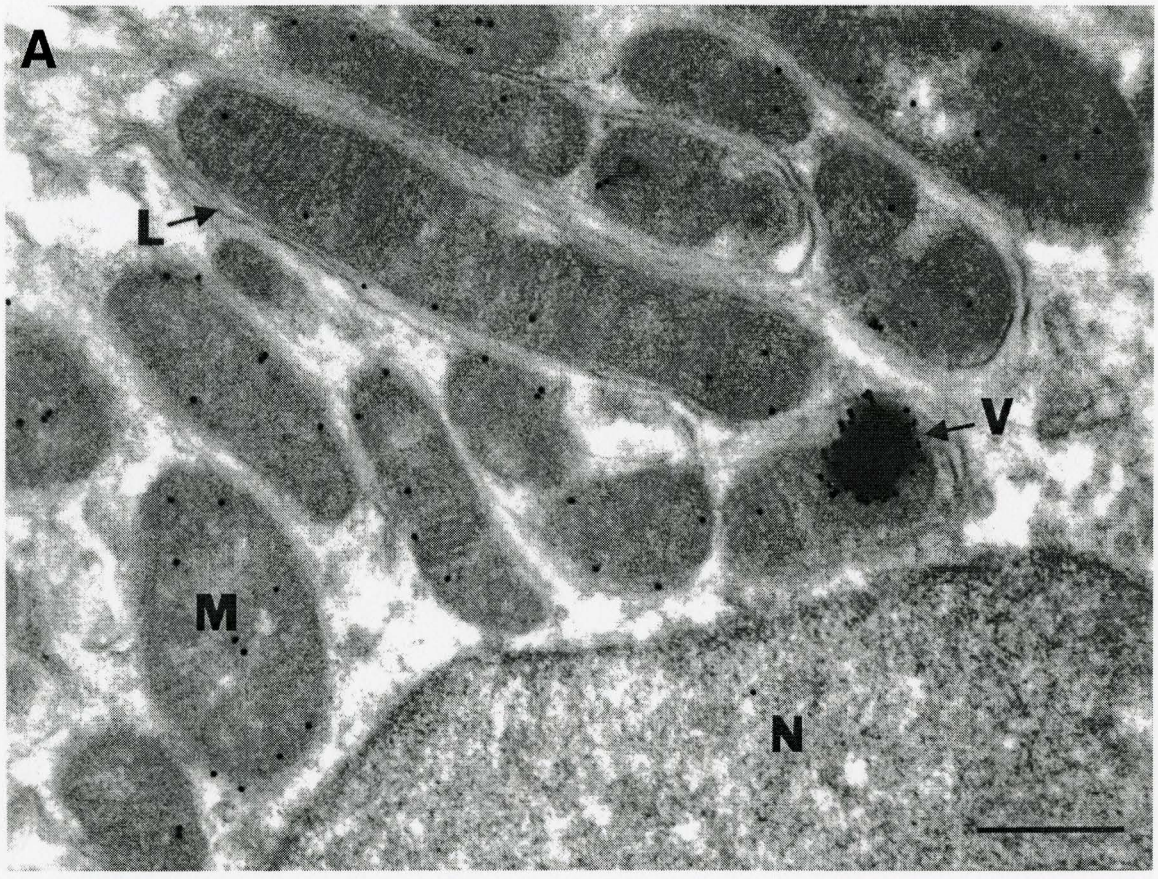

Figure 6A: Extramitochondrial reactivity is seen for mAspAT in distal tubule from rat kidney. In the distal convoluted tubule of rat kidney, labeling is seen in condensing vacuoles as well as in mitochondria. There is no labeling present in cytoplasm or nucleus. Bar = 500 nm. 20 nm gold particles. N, nucleus; M, mitochondria; V, condensing vacuoles; L, lateral intercellular labyrinth.

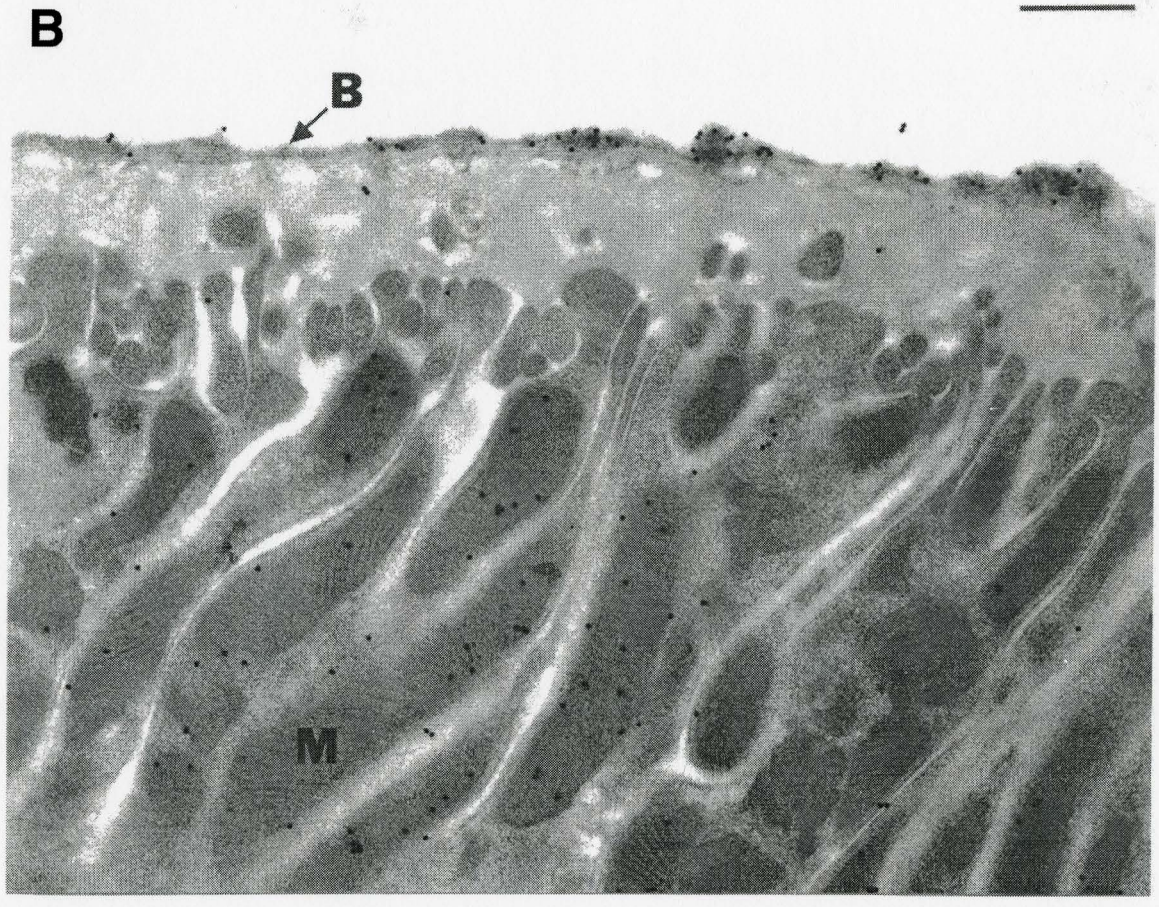

Figure 6B: In the ascending part of thick distal tubule of rat kidney, labeling is seen on the basal plasma membrane in addition to mitochondria. Bar = 500 nm. 20 nm gold particles. M, mitochondria; B, basal membrane.

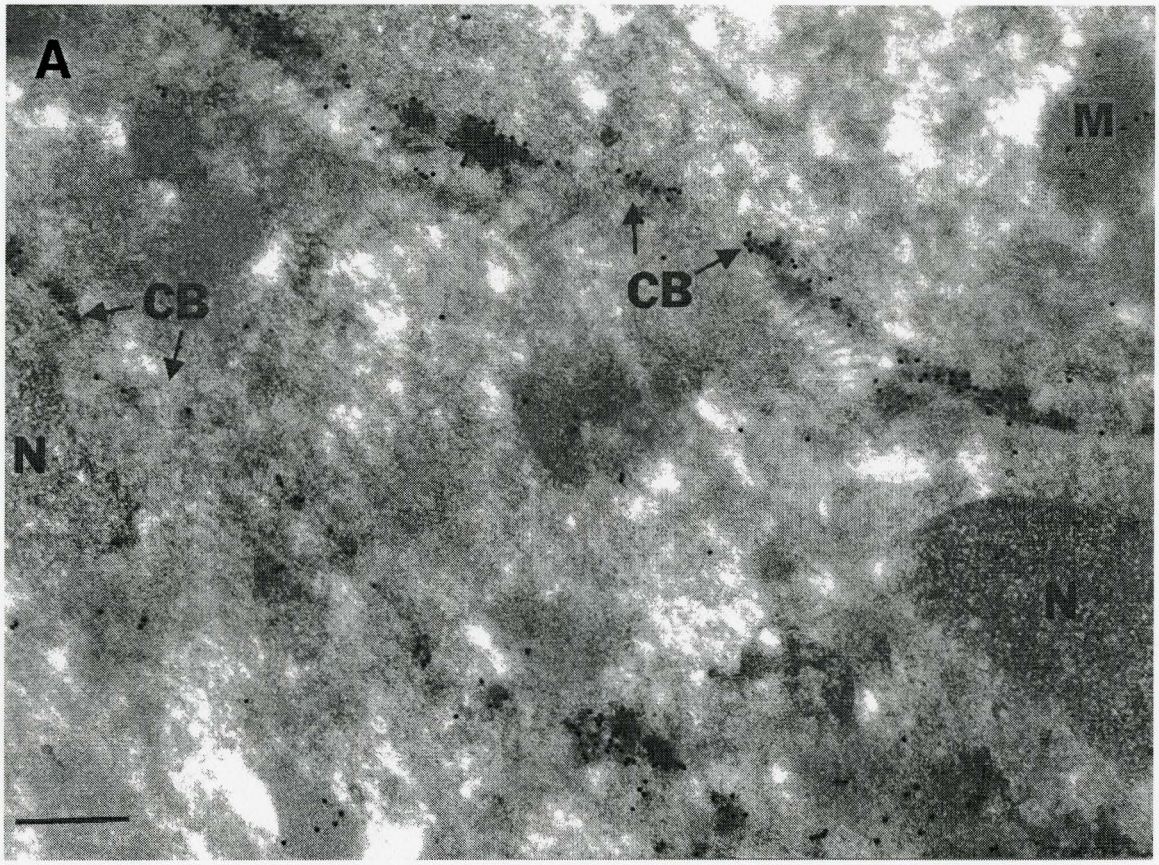

Figure 7A: Surface reactivity for mAspAT in rat kidney. In three adjoining rat kidney cells labeling is seen primarily within mitochondria and along the cell boundaries. Bar = 500 nm. 20 nm gold particles. N, nucleus; M, mitochondria; CB, cell boundary.

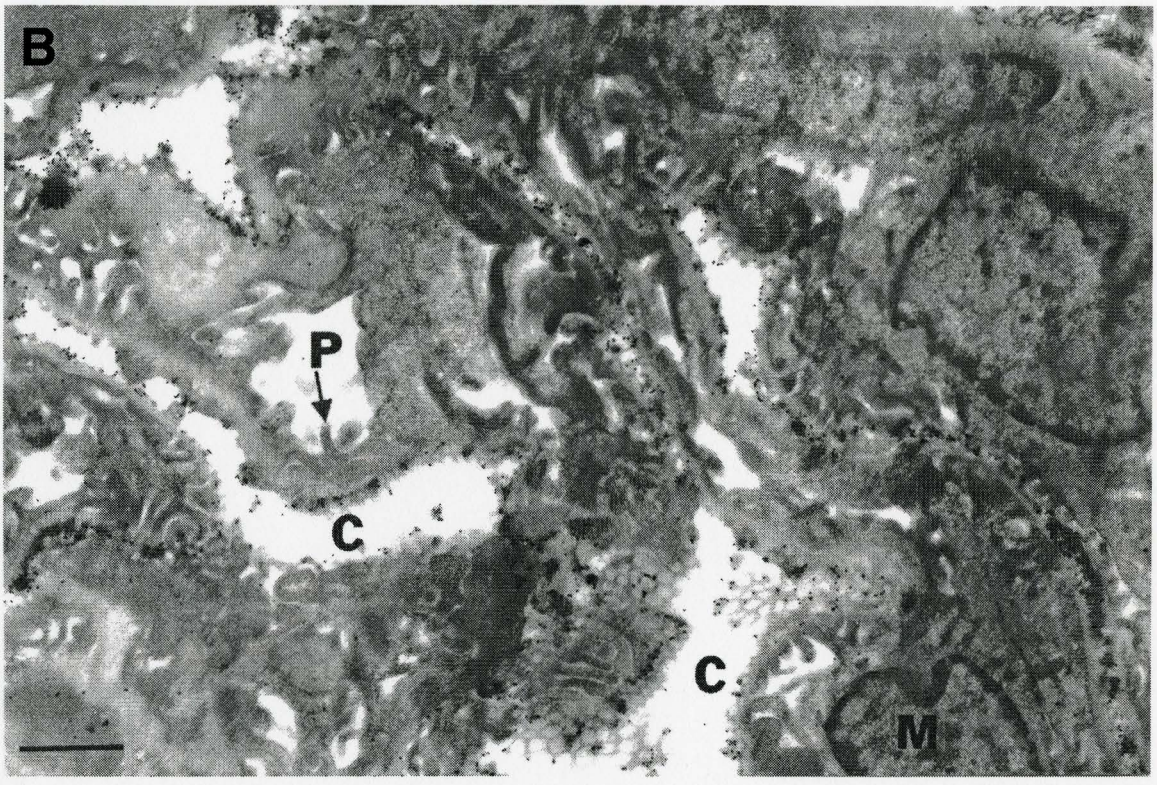

Figure 7B: Surface labeling for mAspAT is seen in rat kidney glomerulus. Labeling is along the luminal side of endothelial cells lining capillaries. Bar = 1000 nm. 20 nm gold particles. C, capillaries; P, pedicels; M, mesangial cells.

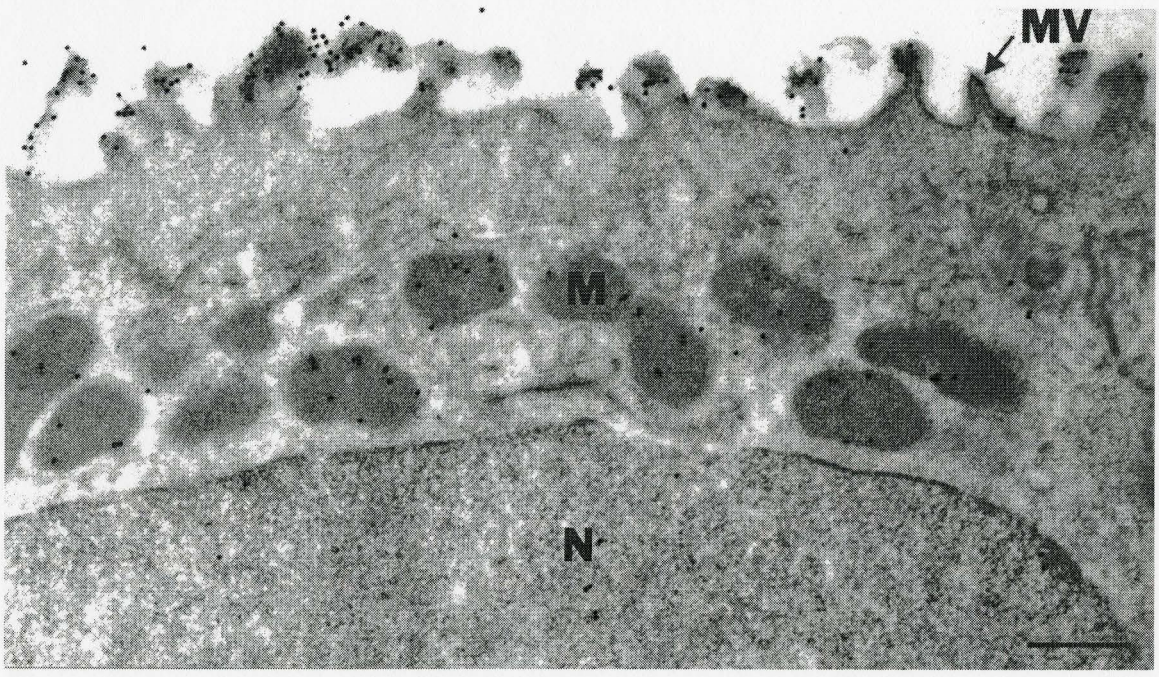

Figure 8: Subcellular localization of mAspAT in rat kidney cortical collecting duct LR Gold sections, using immunogold electron microscopy. Reactivity for mAspAT is seen exclusively in mitochondria and surface microvilli. Bar = 500 nm. 20 nm gold particles. N, nucleus; M, mitochondria; MV, microvilli.

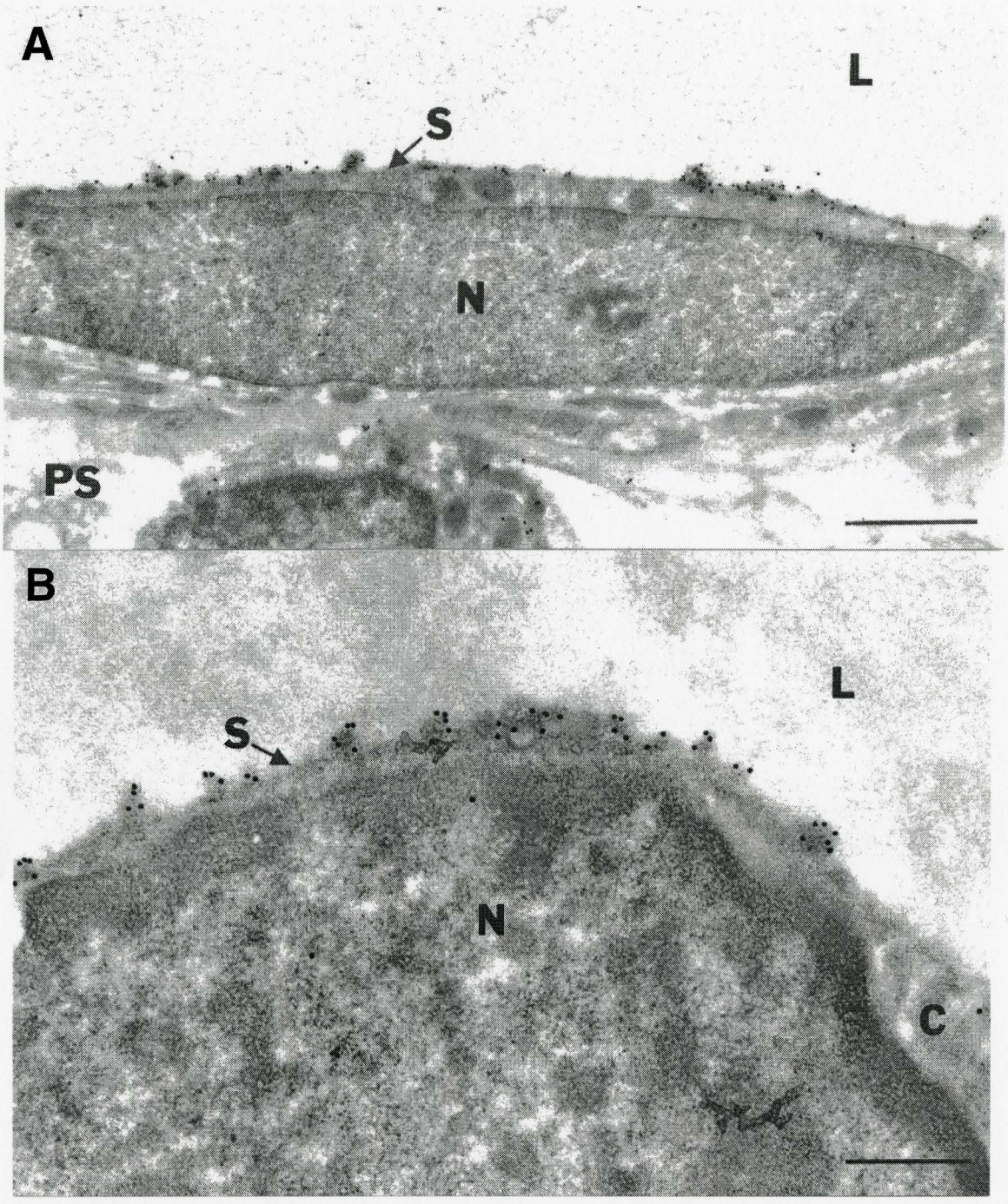

Figure 9: Extramitochondrial localization of mAspAT in the circulatory system. (A) Surface labeling of mAspAT is seen in an endothelial cell lining an arteriole in rat kidney. Bar = 1000 nm. (B) Surface reactivity is seen in a lymphocyte present in a blood vessel from rat kidney. Bar = 500 nm. 20 nm gold particles. N, nucleus; L, lumen; S, surface; PS, pericapillary space; C, cytosol.

Chapter 5.0:

Mitochondrially Encoded Cytochrome c Oxidase Subunit I and II Localizes in Rat Pancreatic Zymogen Granules

5.1 Abstract

Cytochrome c oxidase or COX is a part of the electron transport chain and is involved in transferring electrons from cytochrome c to molecular oxygen coupled with vectorial translocation of protons. In this study, we have used immunogold electron microscopy to study the subcellular localization of COX I and COX II subunits in cultured cells and in a variety of rat tissue sections embedded in LR Gold resin employing monoclonal antibodies raised against human COX I and COX II. Immunofluorescence labeling of BS-C-1 African monkey kidney cells with COX I and COX II showed characteristic mitochondrial labeling. In immunogold labeling studies, the COX I and COX II antibodies showed strong and specific labeling of mitochondria in liver, kidney and pancreas. However, in addition to mitochondrial labeling in rat pancreatic acinar tissue, strong and highly specific labeling with the COX I and COX II antibody was also observed in zymogen granules. The labeling seen in tissues was completely abolished upon omission of the primary COX I and COX II antibodies. These results provide evidence that, similar to a number of other recently described proteins (viz., Hsp60, tumor necrosis factor receptor associated protein 1 (TRAP-1), P32 protein and cytochrome c), COX I and COX II are also found outside mitochondria. Since, COX I and COX II are encoded by mitochondrial DNA, presence of these proteins outside mitochondria provides compelling evidence that they, in fact, exit mitochondria via a specific mechanism(s). These results raise fundamental questions concerning the possible functional roles of mitochondria and mitochondrial resident proteins in diverse

cellular processes in different compartments and the mechanism by which these proteins reach such sites.

5.2 Introduction

Cytochrome c oxidase (COX) or complex IV is present in the inner mitochondrial membrane and is involved in the transfer of electrons from cytochrome c to oxygen, making water (Lehninger et al., 1993). The transfer of electrons is accompanied by the vectorial pumping of protons from the matrix to the intermembrane space. The over all reaction catalyzed by this complex is given by the following equation:

$$4 \text{ Cytochrome } c^{2+} + 8\ H^{+}_{\text{Matrix}} + O_2 \rightarrow 4 \text{ Cytochrome } c^{3+} + 2\ H_2O + 4\ H^{+}_{\text{Intermembrane Space}}$$

Complex IV is about 200 kDa and is composed of 13 heme and copper containing protein subunits, of which 10 are encoded for by the nuclear DNA (Capaldi, 1990). The largest of the subunits COX I, COX II and COX III are transcribed and translated within the mitochondria and form the catalytic core of complex IV (Capaldi, 1990). COX I is composed of 12 transmembrane helices and contains 2 hemes (heme a and heme a_3) and Cu_B. COX II contains a binuclear Cu_A center (Michel et al., 1998). COX III does not contain copper or heme prosthetic groups and is thought to have a structural function (Haltia et al., 1991). Electrons are passed from cytochrome c to the binuclear Cu_A center in COX II, then to heme a, heme a_3 and then reaction with Cu_B results in reduction of molecular oxygen (Tsukihara et al., 1995; Michel et al., 1998).

Mutation in COX I, COX II and COX III gives rise to a variety of unrelated disorders, such as sporadic anaemia, sporadic myopathy and encephalomyopathy (Schon, 2000). Mutations in nuclear encoded proteins involved in the assembly of complex IV are known. Mutation in SURF1 which is involved in the assembly of COX I, COX II and

COX III subunits results in Leigh syndrome, which is characterized by degeneration of brain stem and basal ganglia with elevated lactic acid levels (Yao and Shoubridge, 1999). Mutation in SCO2 which is involved in the insertion of copper centers into subunits, results in cardioencephalomyopathy (Papadopoulou et al., 1999; Schon, 2000). Mutation in COX 10 which is involved in heme formation results in leukodystrophy and tubulopathy (Tzagoloff et al., 1993; Schon, 2000).

In recent years, our lab and other's have shown that a large number of proteins that have been identified based on their extramitochondrial function, have turned to be mitochondrial proteins upon characterization (Soltys and Gupta, 1999; Soltys and Gupta, 2000). These proteins are encoded by a single nuclear gene and there is no evidence for alternate transcription or alternate mRNA translation or splicing. The mechanism by which these nuclear encoded mitochondrial proteins find their way to extramitochondrial sites remains enigmatic and is often attributed to leaky mitochondrial import and mistargeting of the protein.

However, presence of proteins encoded by mitochondrial DNA at specific extramitochondrial sites would exclude most non-specific possibilities and provide compelling evidence that specific mechanisms exist, by which proteins could exit from mitochondria. Therefore, in the present study, we have used high resolution immunogold electron microscopy technique to determine the subcellular localization of COX I and COX II in a variety of normal rat tissue sections embedded in LR Gold resin. In many tissues examined COX I and COX II were mainly localized within mitochondria. However, in rat pancreatic acinar cells, in addition to mitochondria, strong and specific

labeling was also observed in zymogen granules. Interestingly, it has been reported that patients with COX complex deficiency, develop chronic pancreatitis, which is characterized by the premature activation of proenzymes in pancreatic zymogen granules (Kato et al., 1990). The presence of COX I and COX II in zymogen granules raises important questions concerning export mechanism(s) from mitochondria and implications regarding the role of these proteins in different mitochondrial diseases.

5.3 Results

The COX I and COX II antibodies used in the present work is purified mouse monoclonal IgGs antibody against human antigen. The specificity of these antibodies have been previously established by western immunoblot analysis, immunofluorescence and immunohistochemistry (Taanman et al., 1993; Capaldi et al., 1995; Taanman et al., 1996). In western blots of human cell lines COX I and COX II react specifically with one band corresponding to ~ 40 kDa and ~ 25 kDa respectively (Capaldi et al., 1995). In immunofluorescence studies with human cell lines such as 143B and MRC5 fibroblasts, these antibodies show exclusive mitochondrial labeling (Taanman et al., 1996; Marusich et al., 1997).

Subcellular Localization of COX I and COX II in Cultured BS-C-1 Cells

COX I (Figure 1a) and COX II (Figure 1b) antibodies were employed for immunofluorescence labeling of cultured African green monkey kidney, BS-C-1 cells. For both COX I and COX II, the cells show a punctate and bead-string shaped labeling pattern. This labeling pattern is typical of mitochondrial labeling as shown with cells labeled with Hsp60 polyclonal antibody (Figure 1c). From the immunofluorescence studies, there was no indication of extramitochondrial labeling.

Subcellular Localization of COX I and COX II in Normal Rat Tissues

Subcellular localization of COX I and COX II in rat tissue sections were carried out using immunogold electron microscopy. Tissue sections embedded in LR Gold resin were probed with monoclonal antibodies to COX I and COX II, followed by detection with secondary antibodies conjugated to 20 nm gold particles. The specificity of the

secondary immunogold markers was confirmed by carrying out the labeling of tissue sections without the primary antibody. This led to the abolishment of labeling in all compartments suggesting that the immunogold markers do not react with the acrylic resin in which the tissue is embedded.

The results observed in various rat tissue sections are as follows:

Liver: Figure 2 and 3 show labeling pattern of COX I and COX II respectively in rat liver. Reactivity due to COX I in rat liver (Figure 2) was specific for mitochondria with minimal labeling in ER and cytoplasm. Labeling in the nucleus is nearly at background levels. COX II labeling of rat liver shows strong labeling of mitochondria, some labeling was also seen in nucleus, ER and cytosol, but it was at much reduced levels.

Kidney: In rat distal convoluted tubule region of kidney, strong labeling of mitochondria was seen. Figure 4 shows the labeling of COX I in rat kidney. Strong and specific labeling of elongated mitochondria is seen with hardly any labeling present in the cytosol or nucleus. COX II reactivity in rat distal convoluted tubule region of kidney (Figure 5), shows strong labeling of elongated mitochondria which is typical in this region of kidney (Bulger, 1988). There is very little labeling seen in the nucleus, ER and cytosol.

Pancreas: In rat pancreatic acinar cells labeling of COX I (Figure 6) and COX II (Figure 7) were not exclusively localized to mitochondria. Labeling was also observed in zymogen granules and was comparable to the labeling observed in mitochondria. Labeling was also observed in condensing vacuoles which are precursors to zymogen granules (Palade, 1975). The identity of these granules as zymogen granules has been established by labeling rat tissue sections with α-amylase which shows labeling along the

ER, Golgi and zymogen granules (see Figure 5 in Chapter 3). In comparison to α-amylase, COX I and COX II did not show significant labeling of ER or Golgi.

5.4 Discussion

The results presented in this chapter, strongly indicate that COX I and COX II are present within mitochondria in a variety of normal rat tissues and cell lines, which is in accordance with the known function of COX I and COX II in the electron transport chain (Michel et al., 1998). The mitochondrial localization of COX I and COX II is supported by immunofluorescence and immunogold electron microscopic studies. However, the results indicate that in the rat pancreas, in addition to mitochondria, strong labeling was also observed in zymogen granules. In the pancreatic acinar tissue the labeling intensity in zymogen granules was comparable to that seen in mitochondria. However, in comparison to the secretory proteins such as α-amylase, very little labeling with these proteins was seen in ER and Golgi. These results give rise to questions such as: How are these mitochondrially encoded proteins exported? and What are their function(s) in these granules?

It is hard to predict the functional role(s) of COX I and COX II, based on their localization in zymogen granules. However, it is now accepted that one protein, one function is too simplistic and a large number of proteins have been identified to be involved in two or more functions in different compartments (Smalheiser, 1996; Jeffery, 1999; Soltys and Gupta, 1999; Soltys and Gupta, 2000). It is therefore quite possible that COX I and COX II perform a different function(s) in zymogen granules from that in mitochondria, where they comprise the catalytic core of complex IV in the electron transport chain (Michel et al., 1998).

A variety of nuclear encoded mitochondrial proteins have also been shown to be present in zymogen granules and in majority of the cases the function of these proteins in these granules is not known (Cechetto et al., 2000; Cechetto and Gupta, 2000; Soltys et al., 2000; Soltys et al., 2001; Vélez-Granell et al., 1994). Interestingly, it has been reported that patients with COX deficiency could develop chronic pancreatitis (Kato et al., 1990). Pancreatitis is a result of premature activation of proenzymes contained within zymogen granules such as trypsinogen, which leads to activation of other proenzymes, this results in damage to pancreatic acinar cells and eventual death (Kloppel et al., 1986; Scheele et al., 1987). Under normal physiological conditions proenzymes contained within zymogen granules are released into the acinar lumen upon stimulation as inactive proenzymes and are activated in the duodenum (Palade, 1975). Pancreatitis is also characterized by basolateral exocytosis of zymogen granules as opposed to apical exocytosis and results in increased serum amylase and lipase concentrations (Steinberg and Tenner, 1994). It has been suggested that oxidative phosphorylation dysfunction due to COX deficiency could lead to energy deficiency, which could in turn, impair normal pancreatic functioning (Kishnani et al., 1996). But given that COX I and COX II are found in zymogen granules, one cannot rule out the possibility that, impairment of extramitochondrial functioning of these proteins could be involved in pancreatitis.

The current belief is that mitochondria and mitochondrial proteins are primarily involved in functions related to oxidative phosphorylation. These mitochondrial proteins are thought to stay and function only within the mitochondria under normal physiological conditions (Poyton et al., 1992; Soltys and Gupta, 1999; Soltys and Gupta, 2000).

However, a number of proteins that are part of the electron transport chain and proteins involved in oxidative phosphorylation have been shown to be involved in more than one function both within mitochondria and at extramitochondrial sites. Nuclear encoded ATP synthase subunit α has been identified to also function as a molecular chaperone in mitochondria (Luis et al., 1990). ATP synthase subunit α is also present in peroxisomes where its function remains to be elucidated (Cuezva et al., 1990). ATP synthase subunit β, has been reported to occur on the basolateral domain of the hepatocyte plasma membrane and to function as an organic anion binding protein (Wolkoff and Chung, 1980; Bandlow et al., 1998). ATP synthase subunit γ has been shown to be identical to TIM 11 which is a part of the protein translocase machinery present in the mitochondrial innermembrane (Arnold et al., 1998). Subunit I of ubiquinone-cytochrome c oxidoreductase complex in the electron transport chain is also involved in processing of proteins imported into mitochondria (Schulte et al., 1989).

The mechanism by which COX I and COX II get exported to zymogen granules is unclear. Usually the presence of nuclear encoded mitochondrial proteins is attributed to either leaky import, mistargeting, alternate splicing, transcription or translation initiation (Smalheiser, 1996; Soltys and Gupta, 2000). Since COX I and COX II are transcribed and translated within the mitochondria, their presence in zymogen granules, provides strong and compelling evidence that specific mechanism(s) exist by which COX I and COX II are exported. Furthermore, peptides derived from mitochondrially encoded proteins have already been shown to be present and function at extramitochondrial locations. Mitochondrially transmitted factor (MTF) are present on the cell surface as

involved in the export of the N- and C-terminal domains of COX II into the intermembrane space (Hell et al., 1997; Hell et al., 1998), and Pnt 1 is involved in the export of the C-terminal domain of COX II into the intermembrane space (He and Fox, 1999). Given the complexity of export process (Neupert, 1997), there is a strong possibility that additional components of the export machinery will be discovered. Another possibility is that mitochondria which evolved from Gram-negative proteobacteria (Margulis, 1970; Gray and Doolittle, 1982; Margulis, 1993) could have retained a mechanism for protein export and secretion, that has not been discovered yet (Soltys and Gupta, 2000). However it is important to note that no bacterial Sec like proteins are encoded by the yeast genome (Glick and von Heijne, 1996). Other possible mechanisms of export/translocation by which mitochondrial matrix proteins could be localized to specific extramitochondrial compartments are discussed in recent reviews (Soltys and Gupta, 1999; Soltys and Gupta, 2000).

The presence of mitochondrially encoded COX I and COX II in zymogen granules provides compelling evidence that these proteins have been exported/translocated via specific mechanism(s). Although the export mechanism(s) from mitochondria and functional roles of COX I and COX II in zymogen granules remains to be characterized, these results strongly indicate that the cellular functions of mitochondrially encoded proteins are not restricted within mitochondria. Involvement of mitochondrially encoded proteins in extramitochondrial functions should be useful in understanding the puzzling broad spectrum of phenotypes observed in mitochondria related diseases (Wallace, 1999; Schon, 2000).

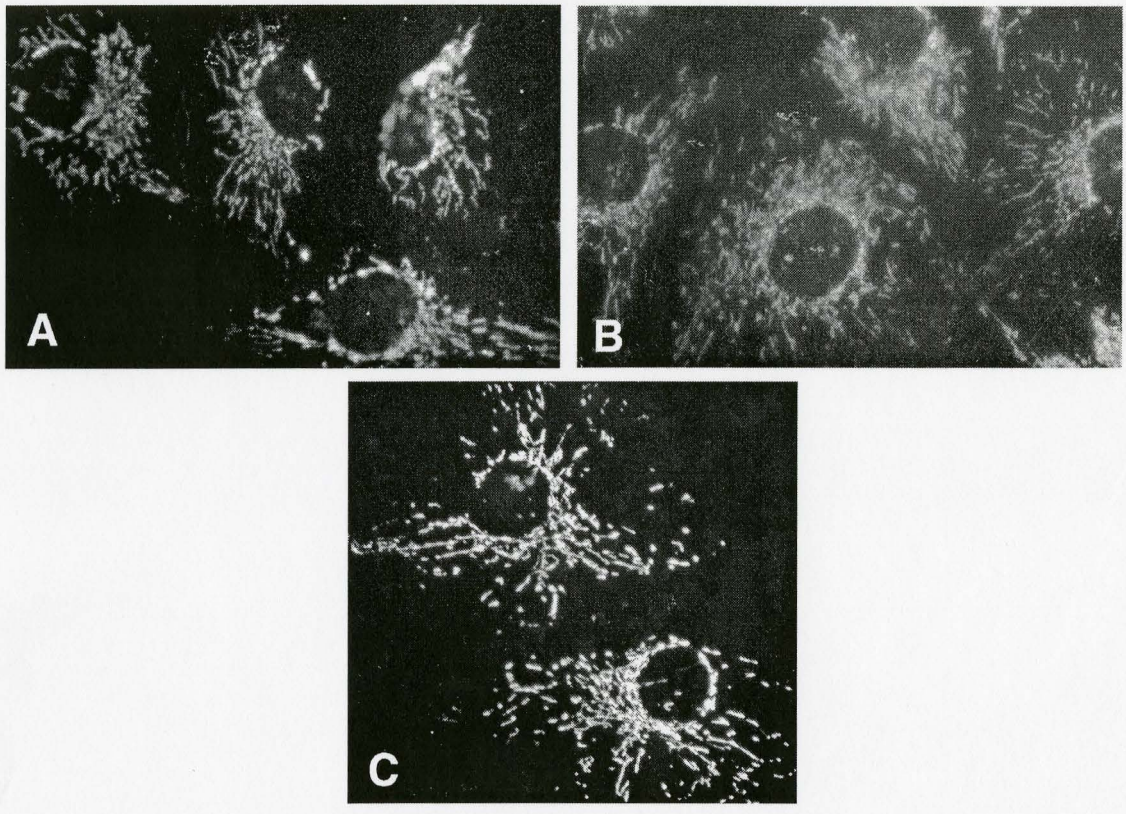

Figure 1: Immunofluorescence labeling of cultured BS-C-1 cells with, (A) mouse monoclonal antibody to COX I, (B) mouse monoclonal antibody to COX II and (C) rabbit polyclonal antibody to Hsp60.

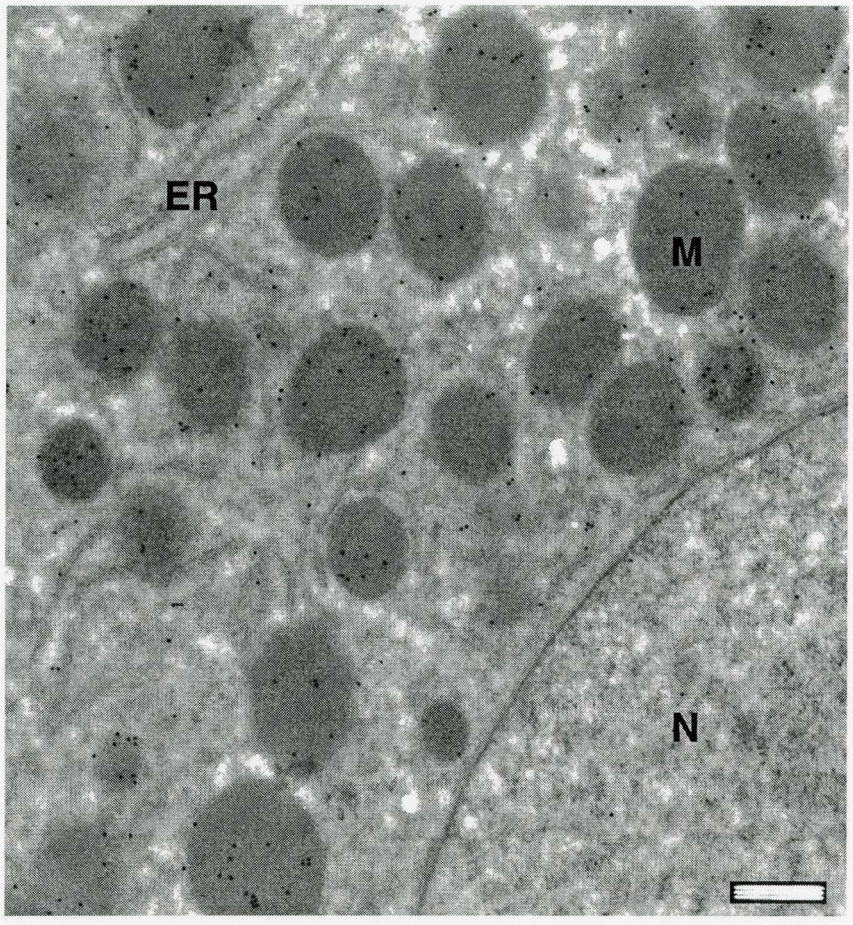

Figure 2: Subcellular localization of COX I in rat liver section. Labeling is seen mainly in mitochondria, with labeling in all other compartments at reduced levels. Immunogold markers conjugated to 20 nm gold particles were used. Bar = 500 nm. M, mitochondria; N, nucleus; ER, endoplasmic reticulum.

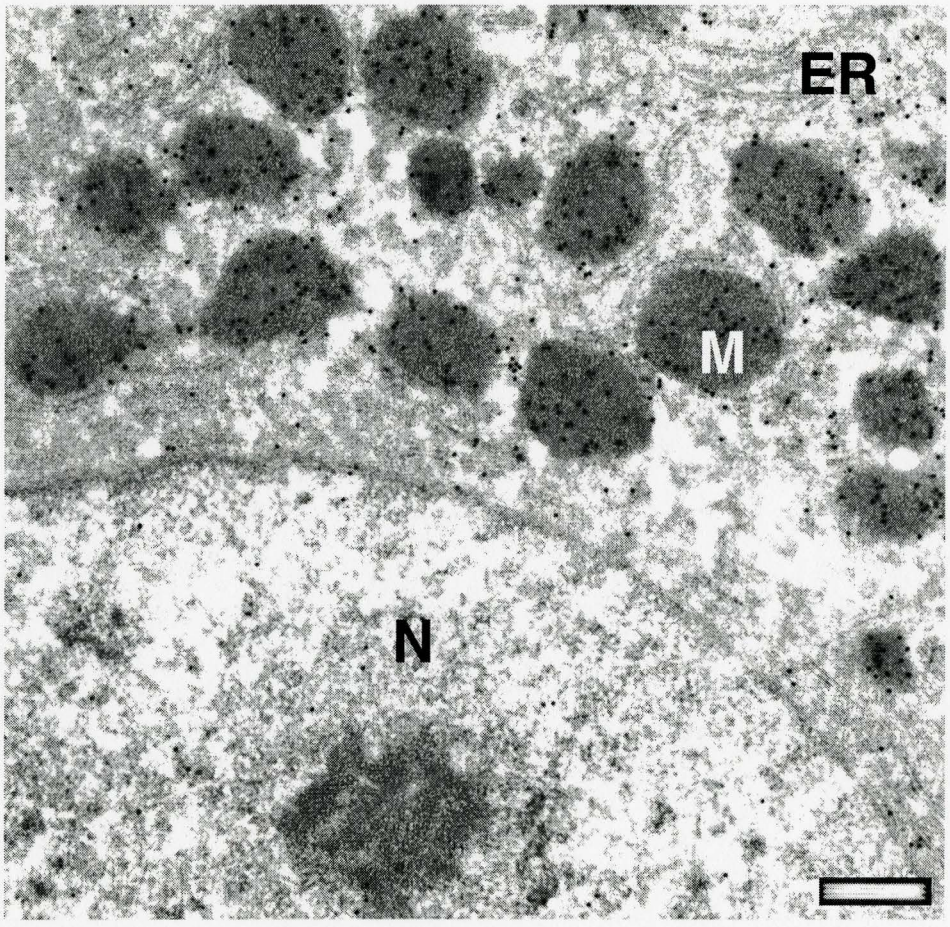

Figure 3: Immunogold localization of COX II in rat liver. Labeling is seen exclusively in mitochondria. 20 nm immunogold markers. Bar = 500 nm. M, mitochondria; ER, endoplasmic reticulum; N, nucleus.

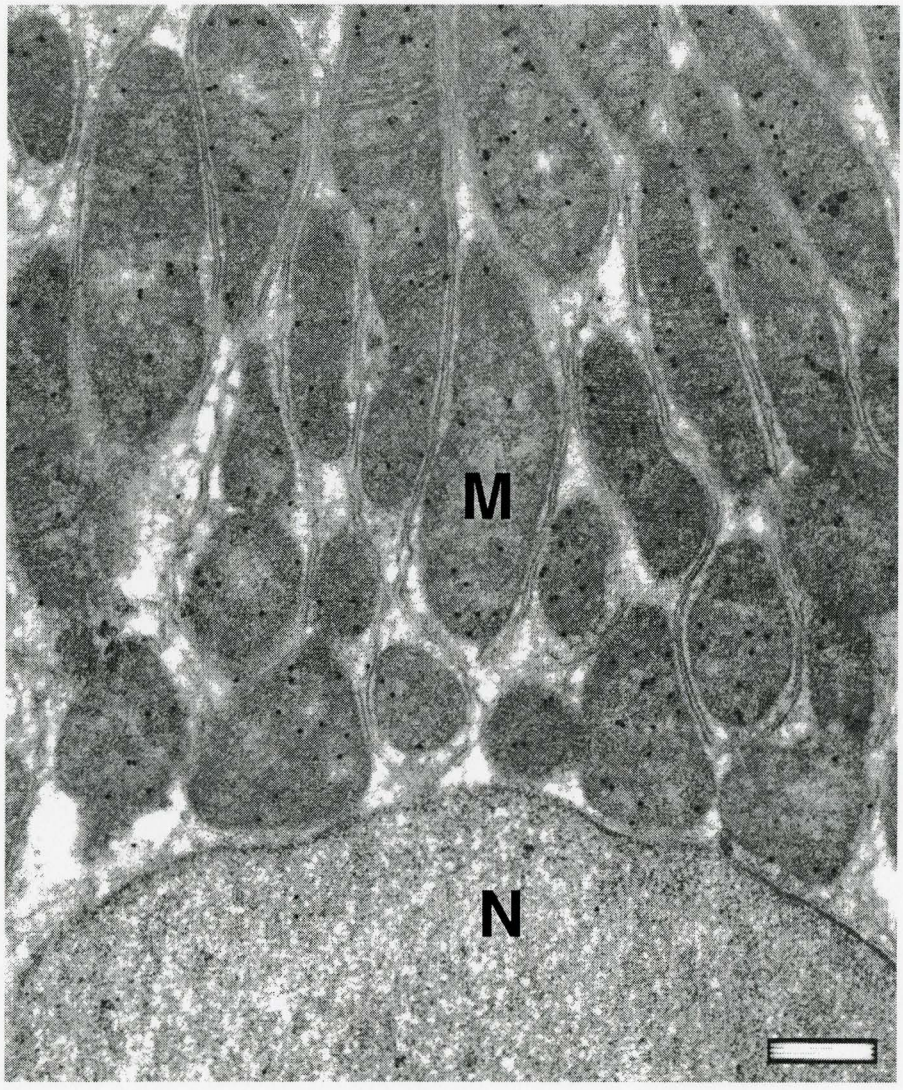

Figure 4: Immunogold localization of COX I in rat distal convoluted tubule region of the kidney. Specific labeling is seen only within mitochondria. 20 nm immunogold markers. Bar = 500 nm. M, mitochondria; N, nucleus.

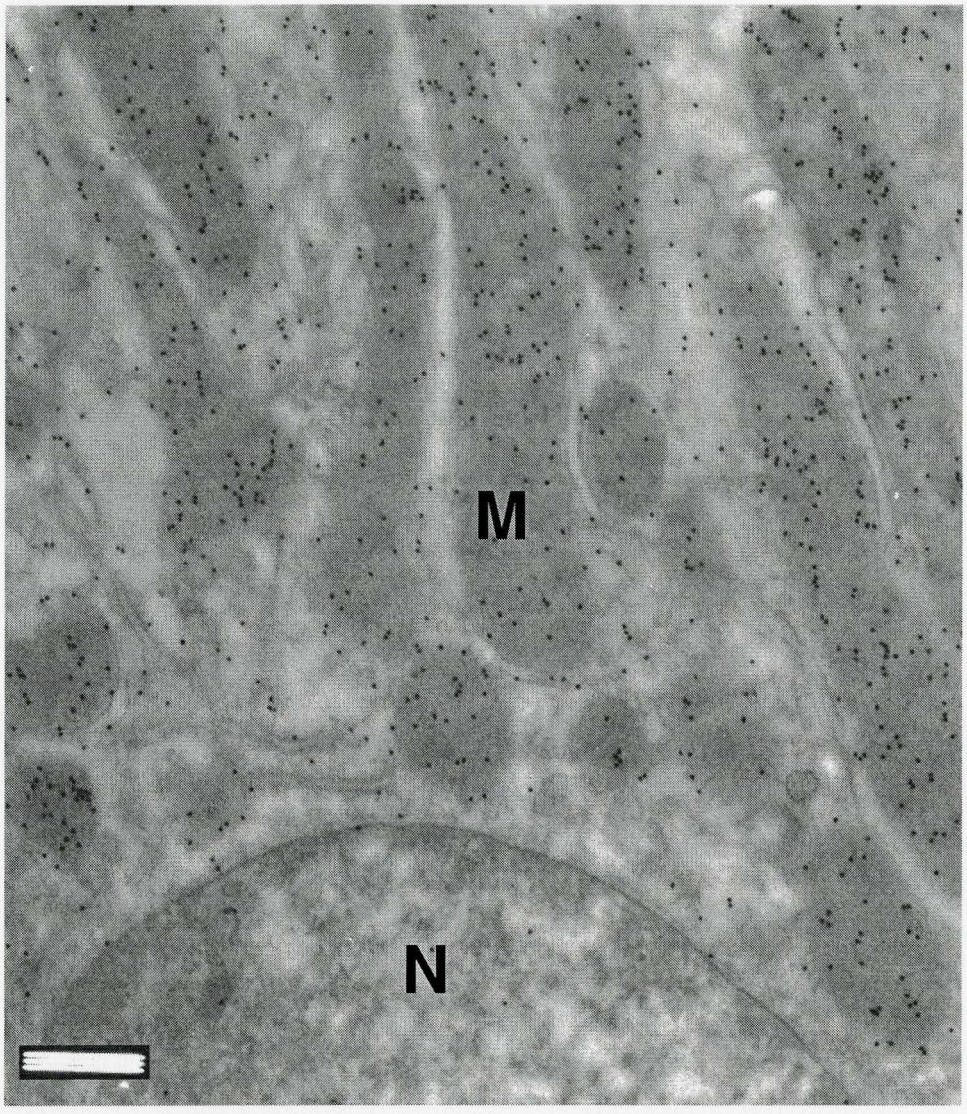

Figure 5: Subcellular localization of COX II in distal convoluted tubule region of rat kidney. Labeling is seen primarily within mitochondria, with labeling seen in all other compartments at reduced levels. 20 nm immunogold markers. Bar = 500 nm. M, mitochondria; N, nucleus.

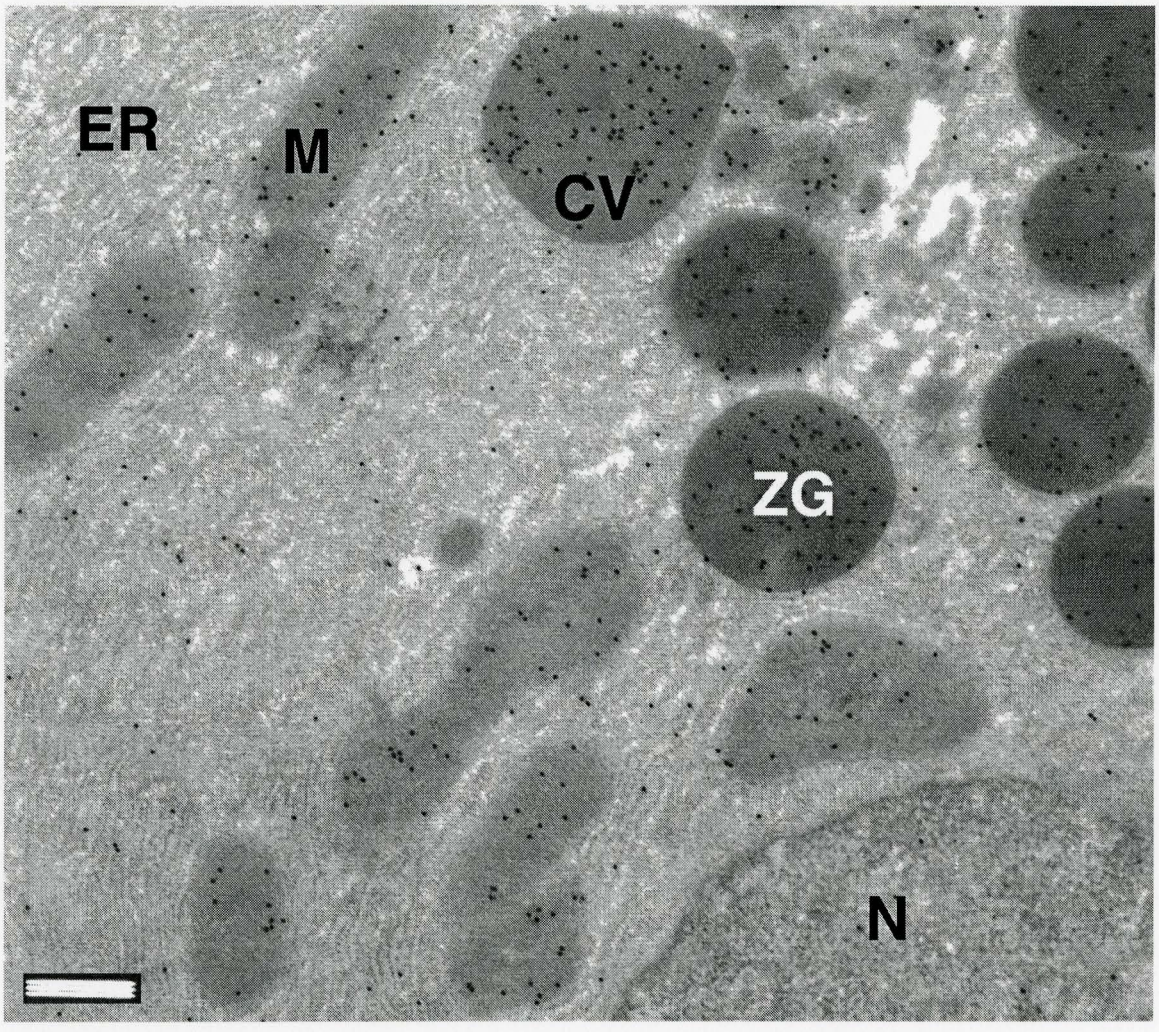

Figure 6: Subcellular localization of COX I in rat pancreatic acinar tissue section. Specific labeling is seen in mitochondria, with extramitochondrial reactivity seen in zymogen granules and condensing vacuole. 20 nm immunogold markers. Bar = 500 nm. M, mitochondria; N, nucleus; ZG, zymogen granule; CV, condensing vacuole; ER, endoplasmic reticulum.

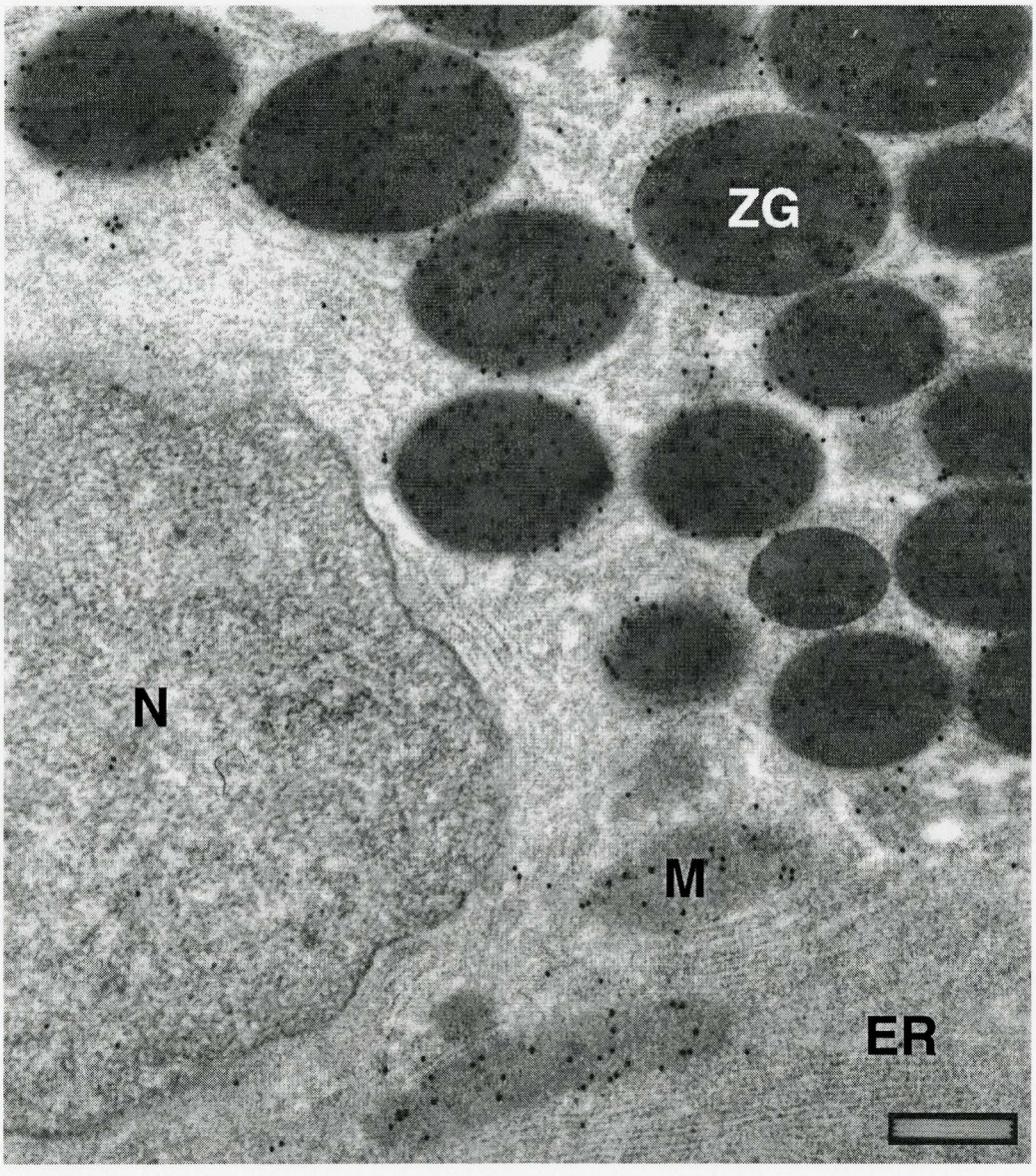

Figure 7: Subcellular localization of COX II in rat pancreatic acinar tissue. Reactivity is seen mainly within mitochondria and zymogen granules. 20 nm immunogold markers. Bar = 500 nm. N, nucleus; M, mitochondria; ZG, zymogen granules; ER, endoplasmic reticulum.

Chapter 6.0:

General Conclusions

The work presented in this thesis, confirms the presence of mitochondrial Chaperonin 10 (Cpn10) and mitochondrial aspartate aminotransferase at extramitochondrial locations in various normal rat tissues. Moreover, the extramitochondrial localization of cytochrome c oxidase subunit I and II (COX I and II), which are encoded by mitochondrial DNA, provides strong evidence that there exists specific mechanism(s) by which mitochondrial proteins are transported/translocated to extramitochondrial sites. The extramitochondrial labeling pattern observed with each antibody in different tissues is unique. For example, Cpn10 in acinar pancreatic tissue shows zymogen granule labeling but in the kidney it does not show any extramitochondrial labeling, whereas mAspAT in acinar pancreas does not label zymogen granules but in the kidney it shows extramitochondrial labeling on the cell surface. This suggests that these mitochondrial proteins are specifically targeted to these extramitochondrial sites and do not appear to be a general process for all proteins. Thus, it is unlikely that the presence of nuclear encoded mitochondrial proteins at extramitochondrial sites, is due to mistargeting or leaky import.

There are a number of reasons that provide strong evidence that the results obtained cannot be due to cross reaction of the antibodies with some other protein at these extramitochondrial sites:

(i) The antibodies used in this work are well characterized and have been used in a number of other studies, by other lab groups. All the antibodies are cross reactive with a single band corresponding to their respective size in western immunoblots (Isola et al.,

1995; Somodevilla-Torres et al., 2000; Capaldi et al., 1995). Immunofluorescent labeling of cultured cell lines with these antibodies, shows exclusive mitochondrial labeling.

(ii) All of the observed labeling with these antibodies, both within mitochondria and in other compartments, was abolished upon omitting the primary antibody in the labeling procedure or upon adsorption of the antibody with purified recombinant protein (done only for Cpn10).

(iii) mAspAT, COX I and COX II are encoded by a single gene. It has also been shown that there is no alternate transcription or alternate mRNA translation or splicing for mAspAT (Bradbury and Berk, 2000). Therefore, it can be said with confidence that these proteins are encoded by a single message. However, it cannot be ruled out that the Cpn10 present at extramitochondrial compartments is encoded by a different nuclear gene or by a differently spliced transcript (Fletcher et al., 2001). It is important to note that based on this work, the identity and molecular size of the cross reactive proteins at these extramitochondrial sites cannot be conclusively established. For this western immunoblot analysis, immunoprecipitation and microsequencing would have to be carried out on purified granules from rat tissue.

The functional roles of these proteins at these extramitochondrial sites are not known and can only be speculated. Cpn10 is found in a variety of secretory granules such as zymogen granules and growth hormone granules, where its functional partner Hsp60 has also been shown to be present (Cechetto et al., 2000). Thus, it is tempting to conclude that Cpn10 functions as a molecular chaperone in these compartments. Cpn10 along with Hsp60 could be involved in the packaging of proteins in these zymogen

granules and/or also function in preventing the early activation of proenzymes in these granules. mAspAT is observed to be present on cell surface in various regions of kidney and is probably involved in its already known extramitochondrial function, as a long chain free fatty acid transporter (Stump et al., 1993). It is quite possible that COX I and COX II which are present in zymogen granules, are involved in novel extramitochondrial functions like Cpn10 and mAspAT.

The precise mechanism(s) by which these mitochondrial proteins are exported is not known, but a number of possibilities have been discussed in recent reviews (Soltys and Gupta, 1999; Soltys and Gupta, 2000). Once these proteins exit mitochondria, they probably reach extramitochondrial sites via a novel pathway because significant amounts of these proteins are not found in ER and Golgi compartments. Further evidence for export comes from the finding that mAspAT isolated from plasma membranes of hepatocytes, adipocytes and cardiac myocytes, has been shown to be the mature form of the protein lacking the N-terminal targeting presequence (Stremmel et al., 1985a; Schwieterman et al., 1988; Berk et al., 1990; Stump et al., 1993). This strongly indicates that mAspAT is derived from the precursor protein which is initially imported and processed in the mitochondria. An analogy of such a process can be derived from an observation that Chinese hamster ovary cells treated with potassium ionophore nonactin, accumulate only precursor Hsp60, while in untreated cells only mature Hsp60 is detectable (Soltys and Gupta, 1996).

Cpn10, mAspAT, COX I and COX II add to the growing list of mitochondrial proteins shown to be present at extramitochondrial sites under normal physiological

conditions. Thus, the current belief that mitochondrial proteins reside and function only within mitochondria, and the role of mitochondrial proteins in normal cellular processes need to be re-examined. Elucidating, the functions and mechanisms by which mitochondrial proteirs reach specific extramitochondrial sites are of great interest and will provide valuable information in understanding the role of these proteins in various cellular processes.

References

Adams J.M. and Cory S. 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281:1322-1326.

Anderson S., Bankier A.T., Barrell B.G., de Bruijn M.H., Coulson A.R., Drouin J., Eperon I.C., Nierlich D.P., Roe B.A., Sanger F., Schreier P.H., Smith A.J., Staden R., and Young I.G. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Antonsson B., Montessuit S., Lauper S., Eskes R., and Martinou J.C. 2000. Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem. J. 345 Pt 2:271-278.

Arnold I., Pfeiffer K., Neupert W., Stuart R.A., and Schagger H. 1998. Yeast mitochondrial F1F0-ATP synthase exists as a dimer: identification of three dimer-specific subunits. EMBO J. 17:7170-7178.

Athanasas-Platsis S., Morton H., Dunglison G.F., and Kaye P.L. 1991. Antibodies to early pregnancy factor retard embryonic development in mice in vivo. J. Reprod. Fertil. 92:443-451.

Ball E.H. and Singer S.J. 1982. Mitochondria are associated with microtubules and not with intermediate filaments in cultured fibroblasts. Proc. Natl. Acad. Sci. USA 79:123-126.

Bandlow W., Strobel G., and Schricker R. 1998. Influence of N-terminal sequence variation on the sorting of major adenylate kinase to the mitochondrial intermembrane space in yeast. Biochem. J. 329 (Pt 2):359-367.

Bandlow W., Strobel G., Zoglowek C., Oechsner U., and Magdolen V. 1988. Yeast adenylate kinase is active simultaneously in mitochondria and cytoplasm and is required for non-fermentative growth. Eur. J. Biochem. 178:451-457.

Beckman K.B. and Ames B.N. 1998. Mitochondrial aging: open questions. Ann. N. Y. Acad. Sci. 854:118-127.

Berger K.H. and Yaffe M.P. 1996. Mitochondrial distribution and inheritance. Experientia 52:1111-1116.

Berk P.D. and Stump D.D. 1999. Mechanisms of cellular uptake of long chain free fatty acids. Mol. Cell Biochem. 192:17-31.

Berk P.D., Wada H., Horio Y., Potter B.J., Sorrentino D., Zhou S.L., Isola L.M., Stump D., Kiang C.L., and Thung S. 1990. Plasma membrane fatty acid-binding protein and mitochondrial glutamic- oxaloacetic transaminase of rat liver are related. Proc. Natl. Acad. Sci. USA 87:3484-3488.

Berthold J., Bauer M.F., Schneider H.C., Klaus C., Dietmeier K., Neupert W., and Brunner M. 1995. The MIM complex mediates preprotein translocation across the mitochondrial inner membrane and couples it to the mt-Hsp70/ATP driving system. Cell 81:1085-1093.

Bhuyan P.K., Young L.L., Lindahl K.F., and Butcher G.W. 1997. Identification of the rat maternally transmitted minor histocompatibility antigen. J. Immunol. 158:3753-3760.

Bradbury M.W. and Berk P.D. 2000. Mitochondrial aspartate aminotransferase: direction of a single protein with two distinct functions to two subcellular sites does not require alternative splicing of the mRNA. Biochem. J. 345 Pt 3:423-427.

Brenner C. and Kroemer G. 2000. Apoptosis. Mitochondria--the death signal integrators. Science 289:1150-1151.

Brown W.M., George M., Jr., and Wilson A.C. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA 76:1967-1971.

Brown W.M., Prager E.M., Wang A., and Wilson A.C. 1982. Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. 18:225-239.

Brudzynski K., Martinez V., and Gupta R.S. 1992. Immunocytochemical localization of heat-shock protein 60-related protein in beta-cell secretory granules and its altered distribution in non-obese diabetic mice. Diabetologia 35:316-324.

Bulger R.E. 1988. The Urinary System. In: Weiss L., editor. Cell and Tissue Biology: A Textbook of Histology. Baltimore: Urban & Schwarzenberg. p 815-850.

Capaldi R.A. 1990. Structure and function of cytochrome c oxidase. Annu. Rev. Biochem. 59:569-596.

Capaldi R.A. 2000. The changing face of mitochondrial research. Trends Biochem. Sci. 25:212-214.

Capaldi R.A., Marusich M.F., and Taanman J. 1995. Mammalian cytochrome-c oxidase: Characterization of enzyme and immunological detection of subunits in tissue extracts and whole cells. Methods Enzymol. 260:117-132.

Cavanagh A.C. 1996. Identification of early pregnancy factor as chaperonin 10: implications for understanding its role. Rev. Reprod. 1:28-32.

Cavanagh A.C. and Morton H. 1994. The purification of early-pregnancy factor to homogeneity from human platelets and identification as chaperonin 10. Eur. J. Biochem. 222:551-560.

Cechetto J.D. and Gupta R.S. 2000. Immunoelectron microscopy provides evidence that tumor necrosis factor receptor-associated protein 1 (TRAP-1) is a mitochondrial protein which also localizes at specific extramitochondrial sites. Exp. Cell Res. 260:30-39.

Cechetto J.D., Soltys B.J., and Gupta R.S. 2000. Localization of mitochondrial 60-kD heat shock chaperonin protein (Hsp60) in pituitary growth hormone secretory granules and pancreatic zymogen granules. J. Histochem. Cytochem. 48:45-56.

Chance B., Sies H., and Boveris A. 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59:527-605.

Chaput M., Claes V., Portetelle D., Cludts I., Cravador A., Burny A., Gras H., and Tartar A. 1988. The neurotrophic factor neuroleukin is 90% homologous with phosphohexose isomerase. Nature 332:454-455.

Chen C., Chen Y., Dai K., Chen P., Riley D.J., and Lee W. 1996. A new member of the hsp90 family of molecular chaperones interacts with the retinoblastoma protein during mitosis and after heat shock. Mol. Cell Biol. 16:4691-4699.

Chirico W.J., Waters M.G., and Blobel G. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 332:805-810.

Christen P. and Metzler D.E. Transaminases. 1985. New York, Wiley. Ref Type: Generic

Corral-Debrinski M., Horton T., Lott M.T., Shoffner J.M., McKee A.C., Beal M.F., Graham B.H., and Wallace D.C. 1994. Marked changes in mitochondrial DNA deletion levels in Alzheimer brains. Genomics 23:471-476.

Craig E.A., Gambill B.D., and Nelson R.J. 1993. Heat shock proteins: molecular chaperones of protein biogenesis. [Review]. Microbiol. Rev. 57:402-414.

Crompton M. 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 341 (Pt 2):233-249.

Cuezva J.M., Santaren J.F., Gonzalez P., Valcarce C., Luis A.M., and Izquierdo J.M. 1990. Immunological detection of the mitochondrial F1-ATPase alpha subunit in the matrix of rat liver peroxisomes. A protein involved in organelle biogenesis? FEBS Lett. 270:71-75.

Dabhi V.M. and Lindahl K.F. 1995. MtDNA-encoded histocompatibility antigens. Methods Enzymol. 260:466-485.

Dahlseid J.N., Lill R., Green J.M., Xu X., Qiu Y., and Pierce S.K. 1994. PBP74, a new member of the mammalian 70-kDa heat shock prtein family, is a mitochondrial protein. Mol. Biol. Cell 5:1265-1275.

Danpure C.J. 1995. How can the products of a single gene be localized to more than one intracellular compartment? Trends Cell Biol. 5:230-238.

Daugas E., Nochy D., Ravagnan L., Loeffler M., Susin S.A., Zamzami N., and Kroemer G. 2000a. Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett. 476:118-123.

Daugas E., Susin S.A., Zamzami N., Ferri K.F., Irinopoulou T., Larochette N., Prevost M., Leber B., Andrews D., Penninger J., and Kroemer G. 2000b. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J. 14:729-739.

Desagher S. and Martinou J.C. 2000. Mitochondria as the central control point of apoptosis. Trends Cell Biol. 10:369-377.

Domanico S.Z., DeNagel D.C., Dahlseid J.N., Green J.M., and Pierce S.K. 1993. Cloning of the gene encoding peptide-binding protein 74 shows that it is a new member of the heat shock protein 70 family. Molecular & Cellular Biology 13:3598-3610.

Edenhofer F., Rieger R., Famulok M., Wendler W., Weiss S., and Winnacker E.L. 1996. Prion protein PrPc interacts with molecular chaperones of the Hsp60 family. J. Virol. 70:4724-4728.

Ellis R.J. 1987. Proteins as molecular chaperones. Nature 328:378-379.

Ellis R.J. and Hartl F.U. 1996. Protein folding in the cell:competing models of chaperonin function. FASEB J. 10:20-26.

Ellis R.J. and van der Vies S.M. 1991. Molecular Chaperones. Annu. Rev. Biochem. 60:321-347.

Estridge M. 1977. Polypeptides similar to the alpha and beta subunits of tubulin are exposed on the neuronal surface. Nature 268:60-63.

Faik P., Walker J.I., Redmill A.A., and Morgan M.J. 1988. Mouse glucose-6-phosphate isomerase and neuroleukin have identical 3' sequences. Nature 332:455-457.

Felts S.J., Owen B.A.L., Nguyen P.M., Trepel J., Donner D.B., and Toft D.O. 2000. The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. J. Biol. Chem. 275:3305-3312.

Fisch P., Malkovsky M., Kovats S., Sturm E., Braakman E., Klein B.S., Voss S.D., Morrissey L.W., DeMars R., Welch W.J., Bolhuis R.L.H., and Sondel P.M. 1990.

Recognition by Human Vτ9/Vδ2 T cells of a Gro EL Homolog on Daudi Burkitt's Lymphoma Cells. Science 250:1269-1273.

Fischer-Lindahl K.E., Hermel B.E., Loveland B.E., and Wang C.-R. 1991. Maternally transmitted antigen of mice: a model transplantation antigen. Annu. Rev. Immunol. 9:351-372.

Fletcher B.H., Cassady A.I., Summers K.M., and Cavanagh A.C. 2001. The murine chaperonin 10 gene family contains an intronless, putative gene for early pregnancy factor, Cpn10-rs1. Mamm. Genome 12:133-140.

Fosslien E. 2001. Mitochondrial medicine--molecular pathology of defective oxidative phosphorylation. Ann. Clin. Lab Sci. 31:25-67.

Gaume B., Klaus C., Ungermann C., Guiard B., Neupert W., and Brunner M. 1998. Unfolding of preproteins upon import into mitochondria. EMBO J. 17:6497-6507.

Georgopoulos C. and Welch W.J. 1993. Role of the major heat shock proteins as molecular chaperones. Annu. Rev. Cell Biol. 9:601-634.

Ghebrehiwet B., Lim B.L., Peerschke E.I., Willis A.C., and Reid K.B. 1994. Isolation, cDNA cloning, and overexpression of a 33-kD cell surface glycoprotein that binds to the globular "heads" of C1q. J. Exp. Med. 179:1809-1821.

Glick B.S. and von Heijne G. 1996. Saccharomyces cerevisiae mitochondria lack a bacterial-type sec machinery. Protein Sci. 5:2651-2652.

Goeser T., Nakata K., Braly L.F., Sosiak A., Campbell C.G., Dermietzel R., Novikoff P.M., Stockert R.J., Burk R.D., and Wolkoff A.W. 1990. The rat hepatocyte plasma membrane organic anion binding protein is immunologically related to the mitochondrial F1 adenosine triphosphatase beta-subunit. J. Clin. Invest. 86:220-227.

Goto Y., Nonaka I., and Horai S. 1990. A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348:651-653.

Gray M.W. 1992. The endosymbiont hypothesis revisited. Int. Rev. Cytol. 141:233-357.

Gray M.W., Burger G., and Lang B.F. 1999. Mitochondrial evolution. Science 283:1476-1481.

Gray M.W. and Doolittle W.F. 1982. Has the endosymbiont hypothesis been proven? Microbiol. Rev. 46:1-42.

Green D.R. and Reed J.C. 1998. Mitochondria and apoptosis. Science 281:1309-1312.

Gupta R.S. 1990. Mitochondria, molecular chaperone proteins and the in vivo assembly of microtubules. Trends Biochem. Sci 15:415-418.

Gupta R.S., Nallur R., and Singh B. 1991. Mammalian cell mutants affected in the major molecular chaperone proteins: Implications for *in vivo* microtubule assembly process (Abstract). In: Stress Proteins and the Heat Shock Response. Cold Spring Harbor, New York. p 151.

Gupta R.S. 1995. Evolution of the chaperonin families (hsp60, hsp10 and Tcp-1) of proteins and the origin of eukaryotic cells. Mol. Microbiol. 15:1-11.

Gupta R.S. and Dudani A.K. 1987. Mitochondrial binding of a protein affected in a mutant resistant to the microtubule inhibitor podophyllotoxin. Eur. J. Cell Biol. 44:278-285.

Gupta R.S. and Gupta R. 1984. Mutants of chinese hamster ovary cells affected in two different microtubule-associated proteins. Genetic and biochemical studies. J. Biol. Chem. 259:1882-1890.

Gupta R.S., Ho T.K., Moffat M.R., and Gupta R. 1982. Podophyllotoxin-resistant mutants of Chinese hamster ovary cells. Alteration in a microtubule-associated protein. J. Biol. Chem. 257:1071-1078.

Gupta R.S., Venner T.J., and Chopra A. 1985. Genetic and biochemical studies with mutants of mammalian cells affected in microtubule-related proteins other than tubulin: mitochondrial localization of a microtubule-related protein. Can. J. Biochem. Cell Biol. 63:489-502.

Gutteridge J.M. 1994. Biological origin of free radicals, and mechanisms of antioxidant protection. Chem. Biol. Interact. 91:133-140.

Haltia T., Saraste M., and Wikstrom M. 1991. Subunit III of cytochrome c oxidase is not involved in proton translocation: a site-directed mutagenesis study. EMBO J. 10:2015-2021.

Hammen P.K. and Weiner H. 1998. Mitochondrial leader sequences: structural similarities and sequence differences. J. Exp. Zool. 282:280-283.

Hartl F.U. 1996. Molecular chaperones in cellular protein folding. Nature 381:571-580.

He S. and Fox T.D 1999. Mutations affecting a yeast mitochondrial inner membrane protein, pnt1p, block export of a mitochondrially synthesized fusion protein from the matrix. Mol. Cell Biol. 19:6598-6607.

Heggeness M.H., Simon M., and Singer S.J. 1978. Association of mitochondria with microtubules in cultured cells. Proc. Natl. Acad. Sci. USA 75:3863-3866.

Hell K., Herrmann J., Pratje E., Neupert W., and Stuart R.A. 1997. Oxa1p mediates the export of t. FEBS Lett. 418:367-370.

Hell K., Herrmann J.M., Pratje E., Neupert W., and Stuart R.A. 1998. Oxa1p, an essential component of the N-tail protein export machinery in mitochondria. Proc. Natl. Acad. Sci. USA 95:2250-2255.

Heller R.A. and Kronke M. 1994. Tumor necrosis factor receptor-mediated signaling pathways. J. Cell Biol. 126:5-9.

Hendrick J.P., Hodges P.E., and Rosenberg L.E. 1989. Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: leader peptides cleaved by two matrix proteases share a three-amino acid motif. Proc. Natl. Acad. Sci. USA 86:4056-4060.

Hermann G.J., Thatcher J.W., Mills J.P., Hales K.G., Fuller M.T., Nunnari J., and Shaw J.M. 1998. Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. J. Cell Biol. 143:359-372.

Holt I.J., Harding A.E., and Morgan-Hughes J.A. 1988. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 331:717-719.

Holt I.J., Harding A.E., Petty R.K., and Morgan-Hughes J.A. 1990. A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. Am. J. Hum. Genet. 46:428-433.

Horst M., Oppliger W., Feifel B., Schatz G., and Glick B.S. 1996. The mitochondrial protein import motor: dissociation of mitochondrial hsp70 from its membrane anchor requires ATP binding rather than ATP hydrolysis. Protein Sci. 5:759-767.

Horton T.M., Graham B.H., Corral-Debrinski M., Shoffner J.M., Kaufman A.E., Beal M.F., and Wallace D.C. 1995. Marked increase in mitochondrial DNA deletion levels in the cerebral cortex of Huntington's disease patients. Neurology 45:1879-1883.

Ikawa S. and Weinberg R.A. 1992. An interaction between p21ras aand heat shock protein hsp60, a chaperonin. Proc. Natl. Acad. Sci. USA 89:2012-2016.

Isola L.M., Zhou S.-L., Kiang C.-L., Stump D.D., Bradbury M.W., and Berk P.D. 1995. 3T3 fibroblasts transfected with a cDNA for mitochondrial aspartate aminotransferase express plasma membrane fatty acid-binding protein and saturable fatty acid uptake. Proc. Natl. Acad. Sci. USA 92:9866-9870.

Jeffery C.J. 1999. Moonlighting proteins. Trends Biochem. Sci. 24:8-11.

Jenuth J.P., Peterson A.C., Fu K., and Shoubridge E.A. 1996. Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. Nat. Genet. 14:146-151.

Jin H., May M., Tranebjaerg L., Kendall E., Fontan G., Jackson J., Subramony S.H., Arena F., Lubs H., Smith S., Stevenson R., Schwartz C., and Vetrie D. 1996. A novel X-linked gene, DDP, shows mutations in families with deafness (DFN-1), dystonia, mental deficiency and blindness. Nat. Genet. 14:177-180.

Joh T., Nomiyama H., Maeda S., Shimada K., and Morino Y. 1985. Cloning and sequence analysis of a cDNA encoding porcine mitochondrial aspartate aminotransferase precursor. Proc. Natl. Acad. Sci. USA 82:6065-6069.

Jones M., Gupta R.S., and Englesberg E. 1994. Enhancement in amount of P1 (hsp60) in mutants of Chinese hamster ovary (CHO-K1) cells exhibiting increases in the A system of amino acid transport. Proc. Natl. Acad. Sci. USA 91:858-862.

Kato S., Miyabayashi S., Ohi R., Nakagawa H., Abe J., Yamamoto K., Watanabe S., and Tada K. 1990. Chronic pancreatitis in muscular cytochrome c oxidase deficiency. J. Pediatr. Gastroenterol Nutr. 11:549-552.

Kaur I., Voss S.D., Gupta R.S., Schell K., Fisch P., and Sondel P.M. 1993. Human peripheral gamma delta T cells recognize hsp60 molecules on Daudi Burkitt's lymphoma cells. J. Immunol. 150:2046-2055.

Khan I.U., Wallin R., Gupta R.S., and Kammer G.M. 1998. Protein kinase A- catalyzed phosphorylation of heat shock protein 60 chaperone regulates its attachment to histone 2B in the T lymphocyte plasma membrane. Proc. Natl. Acad. Sci. USA 95:10425-10430.

Kishnani P.S., Van Hove J.L., Shoffner J.S., Kaufman A., Bossen E.H., and Kahler S.G. 1996. Acute pancreatitis in an infant with lactic acidosis and a mutation at nucleotide 3243 in the mitochondrial DNA tRNALeu(UUR) gene. Eur. J. Pediatr. 155:898-903.

Kispal G., Csere P., Prohl C., and Lill R. 1999. The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. EMBO J. 18:3981-3989.

Kloppel G., Dreyer T., Willemer S., Kern H.F., and Adler G. 1986. Human acute pancreatitis: its pathogenesis in the light of immunocytochemical and ultrastructural findings in acinar cells. Virchows Arch. A Pathol. Anat. Histopathol. 409:791-803.

Knox C., Sass E., Neupert W., and Pines O. 1998. Import into mitochondria, folding and retrograde movement of fumarase in yeast. J. Biol. Chem. 273:25587-25593.

Kobayashi S., Amikura R., and Okada M. 1993. Presence of mitochondrial large ribosomal RNA outside mitochondria in germ plasm of Drosophila melanogaster. Science 260:1521-1524.

Koehler C.M. 2000. Protein translocation pathways of the mitochondrion. FEBS Lett. 476:27-31.

Kroemer G., Dallaporta B., and Resche-Rigon M. 1998. The mitochondrial death/life regulator in apoptosis and necrosis. Annu. Rev. Physiol. 60:619-642.

Lain B., Yanez A., Iriarte A., and Martinez-Carrion M. 1998. Aminotransferase variants as probes for the role of the N-terminal region of a mature protein in mitochondrial precursor import and processing. J. Biol. Chem. 273:4406-4415.

Larsson N.G. and Luft R. 1999. Revolution in mitochondrial medicine. FEBS Lett. 455:199-202.

Le Gall I.M. and Bendayan M. 1996. Possible association of chaperonin 60 with secretory proteins in pancreatic acinar cells. J. Histochem. Cytochem. 44:743-749.

Lehninger A.L., Nelson D.L., and Cox M.M. 1993. Principles of Biochemistry. N.Y.: Worth Publishers, Inc.

Leuenberger D., Bally N.A., Schatz G., and Koehler C.M. 1999. Different import pathways through the mitochondrial intermembrane space for inner membrane proteins. EMBO J. 18:4816-4822.

Lindquist S. and Craig E.A. 1988. The heat-shock proteins. Annual Review of Genetics 22:631-677.

Linnane A.W., Marzuki S., Ozawa T., and Tanaka M. 1989. Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases. Lancet 1:642-645.

Lithgow T. 2000. Targeting of proteins to mitochondria. FEBS Lett. 476:22-26.

Loveland B., Wang C.-R., Yonekawa H., Hermel E., and Lindahl K.F. 1990. Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. Cell 60:971-980.

Luis A.M., Alconada A., and Cuezva J.M. 1990. The alpha regulatory subunit of the mitochondrial F1-ATPase complex is a heat-shock protein. Identification of two highly conserved amino acid sequences among the alpha-subunits and molecular chaperones. J. Biol. Chem. 265:7713-7716.

Margulis L. 1970. Origin of eukaryotic cells. New Haven, C.T.: Yale University Press.

Margulis L. 1993. Symbiosis in cell evolution. New York: W.H.Freeman and Company.

Marusich M.F., Robinson B.H., Taanman J.W., Kim S.J., Schillace R., Smith J.L., and Capaldi R.A. 1997. Expression of mtDNA and nDNA encoded respiratory chain proteins in chemically and genetically-derived Rho0 human fibroblasts: a comparison of subunit proteins in normal fibroblasts treated with ethidium bromide and fibroblasts from a patient with mtDNA depletion syndrome. Biochim. Biophys. Acta 1362:145-159.

Mecocci P., MacGarvey U., and Beal M.F. 1994. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. Ann. Neurol. 36:747-751.

Michel H., Behr J., Harrenga A., and Kannt A. 1998. Cytochrome c oxidase: structure and spectroscopy. Annu Rev Biophys Biomol Struct 27:329-356.

Mitchell P. 1979. Keilin's respiratory chain concept and its chemiosmotic consequences. Science 206:1148-1159.

Morton H. 1984. Early pregnancy factor (EPF): A link between fertilization and immunomodulation. Aust J Biol Sci 37:393-407.

Morton H., Hegh V., and Clunie G.J. 1974. Immunosuppression detected in pregnant mice by rosette inhibition test. Nature 249:459-460.

Muhlenhoff U. and Lill R. 2000. Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria. Biochim. Biophys. Acta 1459:370-382.

Murakami H., Pain D., and Blobel G. 1988. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J. Cell Biol. 107:2051-2057.

Muta T., Kang D., Kitajima S., Fujiwara T., and Hamasaki N. 1997. p32 protein, a splicing factor 2-associated protein, is localized in mitochondrial matrix and is functionally important in maintaining oxidative phosphorylation. J. Biol. Chem. 272:24363-24370.

Neupert W. 1997. Protein import into mitochondria. Annu. Rev. Biochem. 66:863-917.

Noonan F.P., Halliday W.J., Morton H., and Clunie G.J. 1979. Early pregnancy factor is immunosuppressive. Nature 278:649-651.

Ortiz R.G., Newman N.J., Shoffner J.M., Kaufman A.E., Koontz D.A., and Wallace D.C. 1993. Variable retinal and neurologic manifestations in patients harboring the mitochondrial DNA 8993 mutation. Arch. Ophthalmol. 111:1525-1530.

Palade G. 1975. Intracellular aspects of the process of protein synthesis. Science 189:347-358.

Papa S. and Skulachev V.P. 1997. Reactive oxygen species, mitochondria, apoptosis and aging. Mol. Cell Biochem. 174:305-319.

Papadopoulou L.C., Sue C.M., Davidson M.M., Tanji K., Nishino I., Sadlock J.E., Krishna S., Walker W., Selby J., Glerum D.M., Coster R.V., Lyon G., Scalais E., Lebel R., Kaplan P., Shanske S., De Vivo D.C., Bonilla E., Hirano M., DiMauro S., and Schon E.A. 1999. Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. Nat. Genet. 23:333-337.

Parsell D.A., Taulien J., and Lindquist S. 1993. The role of heat-shock proteins in thermotolerance. Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences 339; discussion p 285.

Picketts D.J., Mayanil C.S., and Gupta R.S. 1989. Molecular cloning of a Chinese hamster mitochondrial protein related to the "chaperonin" family of bacterial and plant proteins. J Biol. Chem. 264:12001-12008.

Porter A.G. 1999. Protein translocation in apoptosis. Trends Cell Biol. 9:394-401.

Poyton R., Duhl D.M.J., and Clarkson G.H.D. 1992. Protein export from the mitochondrial matrix. Trends Cell Biol. 2:369-375.

Quillen M., Castello C., Krishan A., and Rubin R.W. 1985. Cell surface tubulin in leukemic cells: molecular structure, surface binding, turnover, cell cycle expression, and origin. J. Cell Biol. 101:2345-2354.

Quinn K.A., Athanasas-Platsis S., Wong T.Y., Rolfe B.E., Cavanagh A.C., and Morton H. 1990. Monoclonal antibodies to early pregnancy factor perturb tumour cell growth. Clin. Exp. Immunol. 80:100-108.

Quinn K.A., Cavanagh A.C., Hilyard N.C., McKay D.A., and Morton H. 1994. Early pregnancy factor in liver regeneration after partial hepatectomy in rats: relationship with chaperonin 10. Hepatology 20:1294-1302.

Quinn K.A. and Morton H. 1992. Effect of monoclonal antibodies to early pregnancy factor (EPF) on the in vivo growth of transplantable murine tumours. Cancer Immunol. Immunother. 34:265-271.

Rapaport D., Brunner M., Neupert W., and Westermann B. 1998. Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. J. Biol. Chem. 273:20150-20155.

Robinson B.H. 1998. Human complex I deficiency: clinical spectrum and involvement of oxygen free radicals in the pathogenicity of the defect. Biochim. Biophys. Acta 1364:271-286.

Rolfe B.E. 1982. Detection of fetal wastage. Fertil. Steril. 37:655-660.

Rossignol R., Malgat M., Mazat J.P., and Letellier T. 1999. Threshold effect and tissue specificity. Implication for mitochondrial cytopathies. J. Biol. Chem. 274:33426-33432.

Rubin R., Quillen M., Corcoran J., Ganapathi R., and Krishan A. 1982. Tubulin as a major cell surface protein in human lymphoid cells of leukemic origin. Cancer Res. 42:1384-1389.

Ryan M.T., Naylor D.J., Hoogenraad N.J., and Hoj P.B. 1995. Affinity purification, overexpression, and characterization of chaperonin 10 homologues synthesized with and without N-terminal acetylation. J. Biol. Chem. 270:22037-22043.

Samali A., Cai J., Zhivotovsky B., Jones D.P., and Orrenius S. 1999. Presence of a pre-apoptotic complex of pro-caspase-3, hsp60 and hsp10 in the mitochondrial fraction of jurkat cells. EMBO J. 18:2040-2048.

Sanyal A. and Getz G.S. 1995. Import of transcription factor MTF1 into the yeast mitochondria takes place through an unusual pathway. J. Biol. Chem. 270:11970-11976.

Sawyer D.E. and Van Houten B. 1999. Repair of DNA damage in mitochondria. Mutat. Res. 434:161-176.

Schatz G. 1996. The protein import system of mitochondria. J. Biol. Chem. 271:31763-31766.

Scheele G., Adler G., and Kern H. 1987. Exocytosis occurs at the lateral plasma membrane of the pancreatic acinar cell during supramaximal secretagogue stimulation. Gastroenterology 92:345-353.

Scheffler I.E. 1999. Mitochondria. New York: Wiley.

Schendel S.L., Montal M., and Reed J.C. 1998. Bcl-2 family proteins as ion-channels. Cell Death Differ. 5:372-380.

Schneider H.C., Westermann B., Neupert W., and Brunner M. 1996. The nucleotide exchange factor MGE exerts a key function in the ATP- dependent cycle of mt-Hsp70-Tim44 interaction driving mitochondrial protein import. EMBO J. 15:5796-5803.

Schon E.A. 2000. Mitochondrial genetics and disease. Trends Biochem. Sci. 25:555-560.

Schulte U., Arretz M., Schneider H., Tropschug M., Wachter E., Neupert W., and Weiss H. 1989. A family of mitochondrial proteins involved in bioenergetICS and biogenesis. Nature 339:147-149.

Schwieterman W., Sorrentino D., Potter B.J., Rand J., Kiang C.L., Stump D., and Berk P.D. 1988. Uptake of oleate by isolated rat adipocytes is mediated by a 40-kDa plasma membrane fatty acid binding protein closely related to that in liver and gut. Proc. Natl. Acad. Sci. USA 85:359-363.

Shoffner J.M., Fernhoff P.M., Krawiecki N.S., Caplan D.B., Holt P.J., Koontz D.A., Takei Y., Newman N.J., Ortiz R.G., Polak M., and . 1992. Subacute necrotizing encephalopathy: oxidative phosphorylation defects and the ATPase 6 point mutation. Neurology 42:2168-2174.

Singh B. and Gupta R.S. 1992. Expression of human 60-kD heat shock protein (HSP60 or P1) in Escherichia coli and the development and characterization of corresponding monoclonal antibodies. DNA & Cell Biology 11:489-496.

Singh B., Soltys B.J., Wu Z.C., Patel H.V., Freeman K.B., and Gupta R.S. 1997. Cloning and some novel characteristics of mitochondrial Hsp70 from Chinese hamster cells. Exp. Cell Res. 234:205-216.

Smalheiser N.R. 1996. Proteins in unexpected locations. Mol. Biol. Cell 7:1003-1014.

Soltys B.J., Andrews D.W., Jemmerson R., and Gupta R.S. 2001. Cytochrome-c localizes in secretory granules in pancreas and anterior pituitary. Cell Biol. Int. 25:331-338.

Soltys B.J. and Gupta R.S. 1996. Immunoelectron microscopic localization of the 60-kDa heat shock chaperonin protein (Hsp60) in mammalian cells. Exp. Cell Res. 222:16-27.

Soltys B.J. and Gupta R.S. 1999. Mitochondrial-matrix proteins at unexpected locations: are they exported? Trends Biochem. Sci. 24:174-177.

Soltys B.J. and Gupta R.S. 2000. Mitochondrial proteins at unexpected cellular locations: export of proteins from mitochondria from an evolutionary perspective. Int. Rev. Cytol. 194:133-196.

Soltys B.J., Kang D , and Gupta R.S. 2000. Localization of P32 protein (gC1q-R) in mitochondria and at specific extramitochondrial locations in normal tissues. Histochem. Cell Biol. 114:245-255.

Somodevilla-Torres M.J., Hillyard N.C., Morton H., Alewood D., Halliday J.A., Alewood P.F., Vesey D.A., Walsh M.D., and Cavanagh A.C. 2000. Preparation and characterization of polyclonal antibodies against human chaperonin 10. Cell Stress & chaperones 5:14-20.

Sonderegger P., Jaussi R., Christen P., and Gehring H. 1982. Biosynthesis of aspartate aminotransferases. Both the higher molecular weight precursor of mitochondrial aspartate aminotransferase and the cytosolic isoenzyme are synthesized on free polysomes. J. Biol. Chem. 257:3339-3345.

Song H.Y., Dunbar J D., Zhang Y.X., Guo D., and Donner D.B. 1995. Identification of a protein with homology to hsp90 that binds the type 1 tumor necrosis factor receptor. J. Biol. Chem. 270:3574-3581.

Speth C., Prohaszka Z., Mair M., Stockl G., Zhu X., Jobstl B., Fust G., and Dierich M.P. 1999. A 60 kD heat-shock protein-like molecule interacts with the HIV transmembrane glycoprotein gp41. Mol. Immunol. 36:619-628.

Stein I., Peleg Y., Even-Ram S., and Pines O. 1994. The single translation product of the FUM1 gene (fumarase) is processed in mitochondria before being distributed between the cytosol and mitochondria in Saccharomyces cereevisiae. Mol. Cell Biol. 14:4770-4778.

Steinberg W. and Terner S. 1994. Acute pancreatitis. N. Engl. J. Med. 330:1198-1210.

Stephen R.E. 1986. Membrane tubulin. Biol. Cell 57:95-109.

Stock D., Leslie A.G., and Walker J.E. 1999. Molecular architecture of the rotary motor in ATP synthase. Science 286:1700-1705.

Stremmel W., Lotz G., Strohmeyer G., and Berk P.D. 1985b. Identification, isolation, and partial characterization of a fatty acid binding protein from rat jejunal microvillous membranes. J. Clin. Invest. 75:1068-1076.

Stremmel W., Strohmeyer G., and Berk P.D. 1986. Hepatocellular uptake of oleate is energy dependent, sodium linked, and inhibited by an antibody to a hepatocyte plasma membrane fatty acid binding protein. Proc. Natl. Acad. Sci. USA 83:3584-3588.

Stremmel W., Strohmeyer G., Borchard F., Kochwa S., and Berk P.D. 1985a. Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membranes. Proc. Natl. Acad. Sci USA 82:4-8.

Stump D.D., Zhou S.L., and Berk P.D. 1993. Comparison of plasma membrane FABP and mitochondrial isoform of aspartate aminotransferase from rat liver. Am. J. Physiol. 265:G894-G902.

Summers K.M., Fletcher B.H., Macaranas D.D., Somodevilla-Torres M.J., Murphy R.M., Osborne M.J., Spurr N.K., Cassady A.I., and Cavanagh A.C. 1998. Mapping and characterization of the eukaryotic early pregnancy factor/chaperonin 10 gene family. Somat. Cell. Mol. Genet. 24:315-326.

Summers K.M., Murphy R.M., Webb G.C., Peters G.B., Morton H., Cassady A.I., and Cavanagh A.C. 1996. The human early pregnancy factor/chaperonin 10 gene family. Biochem. Mol. Med. 58:52-58.

Susin S.A., Lorenzo H.K., Zamzami N., Marzo I., Brenner C., Larochette N., Prevost M.C., Alzari P.M., and Kroemer G. 1999b. Mitochondrial release of caspase-2 and -9 during the apoptotic process. J. Exp. Med. 189:381-394.

Susin S.A., Lorenzo H.K., Zamzami N., Marzo I., Jacotot E., Costantini P., Loeffler M., Larochette N., Goodlett D.R., Aebersold R., Siderovski D.P., Penninger J.M., and Kroemer G. 1999a. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441-445.

Susin S.A., Zamzami N., Castedo M., Hirsch T., Marchetti P., Macho A., Daugas E., Geuskens M., and Kroemer G. 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. 184:1331-1341.

Susin S.A., Zamzami N., and Kroemer G. 1998. Mitochondria as regulators of apoptosis: doubt no more. Biochim. Biophys. Acta 1366:151-165.

Sutovsky P., Moreno R.D., Ramalho-Santos J., Dominko T., Simerly C., and Schatten G. 1999. Ubiquitin tag for sperm mitochondria. Nature 402:371-372.

Taanman J., Burton M.D., Marushich M.F., Kennaway N.G., and Capaldi R.A. 1996. Subunit specific monoclonal antibodies show different steady-state levels of various cytochrome-c oxidase subunits in chronic progressive external ophthalmoplegia. Biochem. Biophys. Acta 1315:199-207.

Taanman J.W., Hall R.E., Tang C., Marusich M.F., Kennaway N.G., and Capaldi R.A. 1993. Tissue distribution of cytochrome c oxidase isoforms in mammals. Characterization with monoclonal and polyclonal antibodies. Biochim. Biophys. Acta 1225:95-100.

The Biology of Heat Shock Proteins and Molecular Chaperones. 1997. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Thevenod F., Gasser K.W., and Hopfer U. 1990. Dual modulation of chloride conductance by nucleotides in pancreatic and parotid zymogen granules. Biochem. J. 272:119-126.

Thomson M. 1998. What are guanosine triphosphate-binding proteins doing in mitochondria. Biochim. Biophys. Acta 1403:211-218.

Thornberry N.A. and Lazebnik Y. 1998. Caspases: enemies within. Science 281:1312-1316.

Thorsness P.E. and Fox T.D. 1990. Escape of DNA from mitochondria to the nucleus in *Saccharomyces cerevisiae*. Nature 346:376-379.

Tranebjaerg L., Schwartz C., Eriksen H., Andreasson S., Ponjavic V., Dahl A., Stevenson R.E., May M., Arena F., Barker D., and . 1995. A new X linked recessive deafness syndrome with blindness, dystonia, fractures, and mental deficiency is linked to Xq22. J. Med. Genet. 32:257-263.

Tsukihara T., Aoyama H., Yamashita E., Tomizaki T., Yamaguchi H., Shinzawa-Itoh K., Nakashima R., Yaono R., and Yoshikawa S. 1995. Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 A. Science 269:1069-1074.

Tzagoloff A., Nobrega M., Gorman N., and Sinclair P. 1993. On the functions of the yeast COX10 and COX11 gene products. Biochem. Mol. Biol. Int. 31:593-598.

Ungermann C., Guiard B., Neupert W., and Cyr D.M. 1996. The delta psi- and Hsp70/MIM44-dependent reaction cycle driving early steps of protein import into mitochondria. EMBO J. 15:735-744.

VanBuskirk A., Crump B., Margoliash E., and Pierce S.K. 1989. A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. J. Exp. Med. 170:1799-1809.

Vander Heiden M.G., Chandel N.S., Schumacker P.T., and Thompson C.B. 1999. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol. Cell 3:159-167.

Venner T.J., Singh B., and Gupta R.S. 1990. Nucleotide sequences and novel structural features of human and Chinese hamster hsp60 (chaperonin) gene families. DNA & Cell Biology 9:545-552.

Vélez-Granell C.S., Arias A.E., Torres-Ruíz J.A., and Bendayan M. 1994. Molecular chaperones in pancreatic tissue: The presence of cpn10, cpn60 and hsp70 in distinct compartments along the secretory pathway of the acinar cells. J. Cell. Sci. 107:539-549.

Viitanen P.V., Lorimer G.H., Seetharam R., Gupta R.S., Oppenheim J., Thomas J.O., and Cowan N.J. 1992. Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring. J. Biol. Chem. 267:695-698.

Voisine C., Craig E.A., Zufall N., Von Ahsen O., Pfanner N., and Voos W. 1999. The protein import motor of mitochondria: unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. Cell 97:565-574.

Vyska K., Meyer W., Stremmel W., Notohamiprodjo G., Minami K., Machulla H.J., Gleichmann U., Meyer H., and Korfer R. 1991. Fatty acid uptake in normal human myocardium. Circ. Res. 69:857-870.

Wadhwa R., Kaul S.C., Ikawa Y., and Sugimoto Y. 1993a. Identification of a novel member of mouse hsp70 family. Its association with cellular mortal phenotype. J. Biol. Chem. 268:6615-6621.

Wadhwa R., Kaul S.C., and Mitsui Y. 1994. Cellular mortality to immortalization: Mortalin. Cell Struct. Funct. 19:1-10.

Wadhwa R., Kaul S.C., Sugimoto Y., and Mitsui Y. 1993b. Induction of cellular senescence by transfection of cytosolic mortalin cDNA in NIH 3T3 cells. J. Biol. Chem. 268:22239-22242.

Wallace D.C. 1999. Mitochondrial diseases in man and mouse. Science 283:1482-1488.

Weiss L. 1988. The Blood. In: Weiss L., editor. Cell and Tissue Biology: A Textbook of Histology. Baltimore: Urban & Schwarzenberg. p 425-443.

Weissman J.S., Kashi Y., Fenton W.A., and Horwich A.L. 1994. GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonative forms. Cell 78:693-702.

Winefield C.S., Farnden K.J., Reynolds P.H., and Marshall C.J. 1995. Evolutionary analysis of aspartate aminotransferases. J. Mol. Evol. 40:455-463.

Wolkoff A.W. and Chung C.T. 1980. Identification, purification, and partial characterization of an organic anion binding protein from rat liver cell plasma membrane. J. Clin. Invest. 65:1152-1161.

Wu M. and Tzagoloff A. 1987. Mitochondrial and cytoplasmic fumarases in Saccharomyces cerevisiae are encoded by a single nuclear gene FUM1. J. Biol. Chem. 262:12275-12282.

Xanthoudakis S., Roy S., Rasper D., Hennessey T., Aubin Y., Cassady R., Tawa P., Ruel R., Rosen A., and Nicholson D.W. 1999. Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. EMBO J. 18:2049-2056.

Yaffe M.P. 1999. The machinery of mitochondrial inheritance and behavior. Science 283:1493-1497.

Yaffe M.P., Harata D., Verde F., Eddison M., Toda T., and Nurse P. 1996. Microtubules mediate mitochondrial distribution in fission yeast. Proc. Natl. Acad. Sci. USA 93:11664-11668.

Yao J. and Shoubridge E.A. 1999. Expression and functional analysis of SURF1 in Leigh syndrome patients with cytochrome c oxidase deficiency. Hum. Mol. Genet. 8:2541-2549.

Zeilstra-Ryalls J., Fayet O., and Georgopoulos C. 1991. The universally conserved GroE (Hsp60) chaperonins. Annu. Rev. Microbiol. 454:301-325.

Zhou S.-L., Gordon R.E., Bradbury M., Stump D., Kiang C.-L., and Berk P.D. 1998. Ethanol up-regulates fatty acid uptake and plasma membrane expression and export of mitochondrial aspartate aminotransferase in HepG2 cells. Hepatology 27:1064-1074.