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CHARACTERIZATION OF THE MITOCHONDRIAL ACTIVE-SITE SERINE PROTEIN LACTB:

filaments in the mitochondrial intermembrane space

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals.

I. Liobikas J, **Polianskyte Z**, Speer O, Thompson J, Alakoskela J-M, Peitsaro N, Franck M, Whitehead MA, Kinnunen PJK, Eriksson O.
Expression and purification of the mitochondrial serine protease LACTB as an N-terminal GST fusion protein in *Escherichia coli*.
Prot Exp Purif, 45, 335-342, 2006.

II. Peitsaro N*, **Polianskyte Z*,** Tuimala J, Pörn-Ares I, Liobikas J, Speer O, Lindholm D, Thompson J, Eriksson O.
Evolution of a family of metazoan active-site-serine enzymes from penicillin-binding proteins: a novel facet of the bacterial legacy.
BMC Evolutionary Biology, 8:26, doi:10.1186/1471-2148-8-26, 2008.

III. Polianskyte Z, Peitsaro N, Dapkunas A, Liobikas J, Soliymani R, Lalowski M, Speer O, Seitsonen J, Butcher S, Cereghetti GM, Linder MD, Merckel M, Thompson J, Eriksson O.
LACTB is a novel filament-forming protein localized in mitochondria.
Proc Natl Acad Sci USA, 106, 18960-18965, 2009.

IV. Polianskyte Z, Thompson J, Eriksson O.
Quantitation of the mRNA levels of obesity-linked protein LACTB in various human tissues.
Submitted.

* Equal contribution

In addition, some unpublished data are presented.

ABBREVIATIONS

ATP	adenosine triphosphate
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
ANT	adenine nucleotide translocator
Apaf-1	apoptotic protease activation factor-1
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 homologous antagonist/killer
BCA	bicinchoninic acid
BIR	baculovirus inhibitory repeat domain
cDNA	complementary DNA
CsCl	cesium chloride
C. elegans	*Caenorhabditis elegans*
CO_2	carbon dioxide
dNTP	deoxynucleotide triphosphates
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
EM	electron microscopy
FTIR	fourier transform infrared spectroscopy
GDH	glutamic acid dehydrogenase
GFP	green fluorescent protein
GLT	glutamate transporter
GLU	glucose transporter
GST	glutathione S-transferase
HtrA2	high temperature required protein A2
hLACTB	human LACTB
IAP	inhibitor of apoptosis protein
IBM	inhibitor of apoptosis protein binding motif
IMM	inner mitochondrial membrane
kDa	kilodalton
LACTB	β-lactamase
LPBP	low molecular weight penicillin-binding protein
LRPPR	leucine-rich pentatricopeptide motif-containing protein
MALDI-TOF-MS	matrix-assisted laser desorbtion ionization-time-of-flight mass spectrometry
MRP-L	mitochondrial ribosomal protein-larger subunit
mtDNA	mitochondrial deoxyribonucleic acid
mLACTB	mouse LACTB
mRNA	messenger RNA
PTP	permeability transition pore
RNA	ribonucleic acid
OMM	outer mitochondrial membrane
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PG	peptidoglycan

PVDF	polyvinylidine fluoride
ROS	reactive oxygen species
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Smac	second mitochondria-derived activator of caspases
tBid	truncated Bid
TCA	tricarboxylic acid cycle or Krebs cycle
TIM	inner membrane translocase
TOM	outer membrane translocase
VDAC	voltage-dependent anion channel
2D BN SDS-PAGE	two-dimensional blue native sodium dodecyl sulphate-polyacrylamide gel electrophoresis

ABSTRACT

Mitochondria have evolved from endosymbiotic α-proteobacteria. During the endosymbiotic process the machinery for synthesis of the bacterial peptidoglycan layer was eradicated from the bacterial genome. Peptidoglycan is synthesized and maintained by active-site serine enzymes belonging to the penicillin-binding protein and β-lactamase superfamily. Mammals harbor a conserved penicillin-binding protein and β-lactamase homolog named LACTB. Recently, LACTB was validated *in vivo* to be causative for obesity.

The aim of this thesis was to investigate the phylogeny, structure, biochemistry and cell biology of LACTB in order to elucidate its physiological function. We show that LACTB and its eukaryotic homologs are closely related to low molecular weight penicillin-binding proteins class B from the α-proteobacterial division. We also provide a structural model of LACTB indicating that LACTB shares characteristic features common to all penicillin-binding proteins and β-lactamases. An N-terminal GST-LACTB fusion protein expressed in *E. coli* was recovered in significant quantities, and GST-LACTB was found to be properly folded. We detected that the expression level of LACTB mRNA was higher in high metabolic rate human tissues. We show that LACTB is a soluble protein localized in the mitochondrial intermembrane space. We discovered that the LACTB preprotein underwent proteolytic processing disclosing an N-terminal tetrapeptide motif also found in a set of apoptosis-inducing proteins. Finally, we reveal that LACTB can polymerize to form stable filaments with lengths ranging from twenty to several hundred nanometers.

These results offer novel insight into the evolution of mitochondria. We conclude that LACTB and bacterial penicillin-binding proteins share a common structural function. LACTB filaments define a distinct microdomain in the intermembrane space implying a role for LACTB in the mitochondrial intermembrane space organization and microcompartmentalization. The implications of these findings for our understanding of the regulation of energy metabolism are discussed.

INTRODUCTION

Mitochondria are key organelles essential for oxidative phosphorylation, energy metabolism, assemblage of iron-sulfur clusters and calcium ion homeostasis. In higher organisms, mitochondria also play an important role in the regulation of programmed cell death, apoptosis (Kroemer et al., 2007). Mitochondrial dysfunction and disturbances in the regulation of cell death are associated with diverse diseases including neurodegeneration (Lin and Beal, 2006), cardiomyopathy, cancer and metabolic syndrome (Wallace, 2005).

Mitochondria share several conserved features with their bacterial ancestor, such as a circular DNA, a smaller ribosome, and a double membrane. However, during endosymbiosis early eukaryotes dumped the major component of the bacterial cell wall, the peptidoglycan layer. Peptidoglycan is synthesized and maintained by penicillin-binding proteins and β-lactamases (Macheboeuf et al., 2006). In spite of the lack of peptidoglycan, mammals harbor a protein named LACTB that shares sequence similarity with bacterial penicillin-binding proteins and β-lactamases (Smith et al., 2001).

LACTB is an active-site serine enzyme. In 2005, when this thesis was initiated, only scattered fragments of information were available about LACTB's function. LACTB had been identified in several mitochondrial proteome survey studies (Koc et al., 2001; Taylor et al.; 2003, Mootha et al., 2003), suggesting that LACTB is ubiquitously expressed in mammalian mitochondria. It had been shown that LACTB is regulated at both the transcriptional and posttranslational levels (Kim et al., 2006; Lee et al., 2007). More recent studies demonstrated that LACTB is modulated by an immune response triggered by a viral or fungal infection (He at al., 2006; Johung et al., 2007; Pujol et al., 2008). In skeletal muscle, LACTB mRNA expression is increased by insulin (Rome et al., 2003). Very recently, *Lactb* was validated *in vivo* as an obesity gene (Chen et al., 2008). Gene expression profiling of LACTB transgenic mice revealed that LACTB is associated with fatty acid metabolic pathway (Yang et al., 2009). Altogether, these findings suggest that LACTB is involved in metabolic processes.

Since eukaryotes lack the synthesis machinery for peptidoglycan, the question arises as to what is the biochemical and physiological role(s) of LACTB in metazoans? In this study, bioinformatics analysis, biochemical, proteomic and molecular biology experiments were used to clarify the function of LACTB.

1. LITERATURE OVERVIEW

The focus of this doctoral thesis is LACTB. In the first part of the literature overview the reader will be given a general introduction to the biology of mitochondria: an essential central organelle for cell life and death. In the second part of the literature overview the relation of LACTB with bacterial penicillin-binding proteins and β-lactamases will be described.

1.1. Mitochondria

1.1.1 Evolution of mitochondria

Mitochondria are semiautonomous organelles that have played a prominent role in the evolution of eukaryotic cells (Embley and Martin, 2006; de Duve, 2007). Mitochondria harbor their own genome comprised of a small circular DNA. In addition, the mitochondrial ribosomes and the double membrane resemble the corresponding bacterial ones. Based on these characteristic prokaryotic-like features, mitochondria are believed to have originated through endosymbiosis of ancient bacteria.

Two versions of the endosymbiotic theory have been proposed for the origin of eukaryotic cells (Margulis, 1981; Cavalier-Smith, 2006). According to one version of the endosymbiotic theory, the eukaryotic cell ancestor was an anaerobic autotrophic archaebacterium. This autotrophic archaebacterium utilized hydrogen as its source of energy for ATP production, and either CO_2, methylamine, formic acid or acetate as its carbon source (Martin and Muller, 1998). This version of the endosymbiotic theory postulates that the anaerobic ancestor of the eukaryotic cell acquired a mitochondrion through the capture of an aerobically respiring heterotroph, a purple non-sulphur bacterium, belonging to the α-proteobacterial group (John and Whatley, 1975; Margulis, 1981). In contrast, the other version of the endosymbiotic theory postulates that both host and symbiont were facultative aerobes. A phagotrophic host using oxygen and excreting CO_2 and minerals enslaved a photosynthetic purple non-sulphur α-proteobacteria for CO_2 fixation through phagocytosis. Furthermore, the photosynthetic purple non-sulphur α-proteobacteria contained precursors of mitochondrial cristae, so called chromotophores. The chromotophores are invaginations from the inner membrane that housed respiration and photosynthesis machineries (Cavalier-Smith, 2006). A consequence of the endosymbiotic relationship was the loss of redundant genes and the transfer of genes from the symbiont to the host's nucleus.

During evolution, the symbiont underwent genome size alterations. The symbiont's lost genes were associated with regulation, transport, metabolism and photosynthesis suggesting that the symbiont's ancestor was metabolically highly versatile (Boussau et al., 2004; Cavalier-Smith, 2006). In addition, the symbiont lost genes for the peptidoglycan layer that is synthesized and maintained by penicillin-binding proteins and β-lactamases (Massova and Mobashery, 1998).

Eukaryotic cells are condemned to retain mitochondria, since the cells have become dependent on mitochondria for vital metabolic processes. Mitochondria by contrast rely on their host, since most of the mitochondrial proteins are encoded by nuclear genes.

1.1.2 Mitochondrial structure

Mitochondria range from 1-10 μm in size. The mitochondrial number varies from a single mitochondrion in retinal cell to thousands of mitochondria in hepatocytes. The mitochondrial shape also varies depending on the cell type, from the tubular-shaped mitochondria in many cell types to mitochondria that consist of sheets and have a spherical-shape (Perkins et al., 2001; Scheffler, 2001). A mitochondrion is delimited by a double membrane that divides the mitochondrion into two distinct subcompartments: the intermembrane space and the matrix.

The outer mitochondrial membrane (OMM) is composed of about 50% phospholipids and 50% proteins. The OMM contains an abundant protein, called the voltage-dependent anion channel (VDAC) or porin that under physiological conditions renders the OMM permeable to molecules having molecular mass up to 5 kDa. VDAC belongs to the porin family and has a β-barrel structure like bacterial porins. In this respect, the OMM is reminiscent of the outer membrane of gram-negative bacteria.

The inner mitochondrial membrane (IMM) is composed of about 20% phospholipids and 80% proteins. The IMM contains a phospholipid with four fatty acyl chains, cardiolipin, which renders the inner membrane impermeable to ions and solutes. It is interesting to note that cardiolipin, found in the bacterial plasma membrane, is predominantly localized in IMM with minor traces in OMM (de Kroon et al., 1997; reviewed by Mileykovskaya and Dowhan, 2009). An impermeable inner membrane is essential for the maintenance of the electrochemical potential required to drive mitochondrial ATP synthesis. Various transporter proteins such as inner membrane translocase (TIM) or adenine nucleotide translocase (ANT) embedded in IMM shuttle proteins and metabolites between the matrix and the intermembrane space. The surface area of the IMM is larger than the outer membrane; therefore, the inner membrane forms numerous invaginations or cristae that protrude into the matrix (fig. 1) (reviewed by Zick et al., 2009).

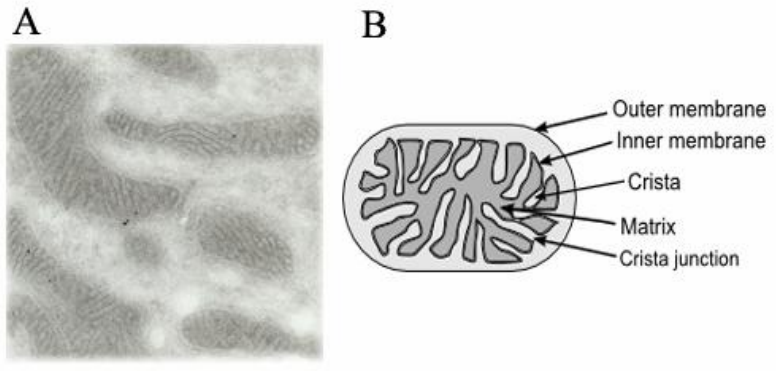

Fig. 1. Image of mitochondria. *(A) Electron micrograph of mitochondria from rat liver tissue. (B) Schematic illustration of mitochondrial structure.*

A recent model of the mitochondrial ultrastructure indicates that the IMM is divided to two distinct subcompartments: the inner boundary membrane and the crista membrane (reviewed by Manella, 2006). Thus, the crista separates the intermembrane space into two parts: the intracristal space and the peripheral intermembrane space (Frey et al., 2002). Electron tomography shows that cristae are connected to the inner boundary membrane forming tubular cristae junctions. Cristae junctions are hypothesized to present diffusion barriers for proteins and molecules such as ATP or ADP from the intracristal space to the peripheral part of the intermembrane space (Frey et al., 2002; Manella, 2006). It has been shown that the ATP synthase dimers induce curvature of the inner membrane (Strauss et al., 2008). Thus, the ATP synthase complex can be involved in shaping the cristae. Both the mitochondrial outer and inner membranes undergo remodeling during programmed cell death. Proteins involved in programmed cell death cause an increase in the diameter of the cristae junctions. The increased diameter of crista junction allows the diffusion of proteins into the peripheral part of the intermembrane space for further release to the cytosol (Scorrano et al., 2002). However, the opposite effect has been observed with cristae junctions narrowing during programmed cell death (Yamaguchi et al., 2008), suggesting that various experimental models have different effects on cristae remodeling. To summarize, the functional consequence of cristae junctions is microcompartmentation of metabolites and macromolecules inside cristae.

The unique ultrastructural architecture of mitochondria creates a platform for their many vital functions.

1.1.3 Mitochondrial function

Mitochondria play a central role in cellular processes such as oxidative phosphorylation, tricarboxylic acid cycle (TCA cycle), β-oxidation of fatty acids, apoptosis, assembly of iron-sulfur clusters and regulation of calcium ion homeostasis.

1.1.3.1 Mitochondria in energy metabolism

Core energy metabolism

Mitochondria are essential for energy production required to maintain cellular functions. The major energy-generating metabolic pathways of cells include glycolysis, fatty acid oxidation, ketone body oxidation and amino acid catabolism.

Glycolysis, the breakdown of glucose and other carbohydrates, generates NADH and a principal mitochondrial substrate, pyruvate. Pyruvate is further oxidized in the mitochondrial matrix to produce NADH and acetyl-CoA. Carbohydrates represent the main energy source in humans, e.g. the brain uses only glucose under normal conditions. Fatty acid oxidation or β-oxidation also generates acetyl-CoA, NADH and $FADH_2$. Fatty acids constitute a major fuel, particularly in resting skeletal muscle and heart. Fatty acid

oxidation intermediates, ketone bodies, are important sources of acetyl-CoA molecules. During a prolonged starvation or in diabetes, the brain uses ketone bodies as energy. In addition, amino acids provide energy for mitochondria. Skeletal muscle uses amino acids as a source of fuel during prolonged starvation and exercise. Amino acids are degraded to pyruvate, acetyl-CoA, acetoacetate, or intermediates of the TCA cycle.

The TCA cycle, comprised of enzyme-catalyzed reactions, involves the final oxidation of metabolites deriving from carbohydrates, fatty acids, and amino acids. The acetyl-CoA derived from glycolysis, fatty acid oxidation and ketone body oxidation serves as the source of energy for the TCA cycle. The NADH and $FADH_2$ produced in the TCA cycle and in the energy-generating metabolic pathways are donated to the mitochondrial electron transport chain (Schapira, 2002; Nelson and Cox, 2008).

Mitochondrial electron transport chain

The mitochondrial electron transport chain or the respiratory chain is composed of four multiprotein enzyme complexes and two electron-carries embedded in the IMM (Schapira, 2002). The mitochondrial respiratory chain mediates the electron transfer from NADH or $FADH_2$ to the final acceptor, molecular oxygen, producing water. Electron transport is coupled to the pumping of protons across the IMM into the intermembrane space, resulting in the generation of a proton-motive force. This proton-motive force is a combination of the proton concentration gradient (ΔpH) and the membrane electrochemical gradient ($\Delta\psi_m$) (Mitchell, 1961). The movement of protons back into the matrix, down their concentration gradient, is coupled to the phosphorylation of ADP to ATP by complex V (fig. 2). The process, in which ATP is formed as a result of electron transfer from NADH or $FADH_2$ to oxygen by respiratory chain, is known as oxidative phosphorylation.

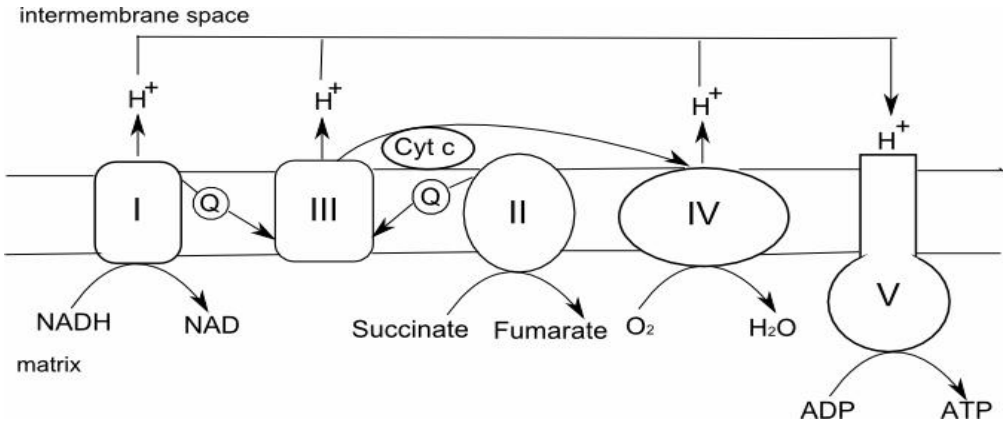

Fig. 2. Schematic illustration of the mitochondrial electron transport chain and ATP synthesis*. The four complexes of the electron transport chain include:* ***complex I****, NADH-dehydrogenase,* ***complex II****, succinate dehydrogenase,* ***complex III****, ubiquinol-cytochrome c reductase,* ***complex IV****, cytochrome c oxidase, and* ***complex V,*** *ATP synthase. Cytochrome c and coenzyme Q reside in the IMM and function as an electron shuttles.*

Uncoupling of oxidative phosphorylation results when substrate oxidation by the mitochondrial respiratory chain is not coupled to phosphorylation. The consequence of

uncoupling is the production of heat instead of ATP. Uncoupling proteins (UCPs) can leak protons across the IMM, and hence uncouple the oxidative phosphorylation causing a decrease in ATP production (Harper et al., 2004). Single electrons can escape from the mitochondrial respiration chain leading to the formation of reactive oxygen species (ROS).

Respiratory chain complexes form large supermolecular assemblies called respiratory supercomplexes or respirasomes that may function for efficient electron transport during respiration (Acín-Pérez et al., 2008).

1.1.3.2 Other functions of mitochondria

Urea cycle

Mitochondria play a role in the urea cycle. Within the urea cycle excess nitrogen is converted into urea. Two reactions of the urea cycle take place in mitochondria: (i) carbomoyl phosphate formation from ammonia and (ii) citrulline formation from carbamoyl phosphate and ornithine.

Biosynthetic reactions

Mitochondria are vital for several biosynthetic pathways, including steroid, heme, and iron-sulfur cluster synthesis (Scheffler, 2001; Nelson and Cox, 2008).

Cholesterol is a steroid that modulates the fluidity of biological membranes. Furthermore, cholesterol is the precursor of steroid hormones that regulate metabolism, growth and developmental processes. The steroid synthesis pathway is initiated and completed in mitochondria with some intermediate reactions taking place in the cytosol. Similarly to the steroid synthesis pathway, several reactions of the heme synthesis pathway take place in mitochondria. The amino acid glycine and the TCA cycle intermediate succinyl CoA are building blocks for porphyrin synthesis. The addition of iron to the center of the porphyrin ring produces heme, a prosthetic group in proteins such as hemoglobin, catalase, peroxidase, and cytochrome c. Iron-sulfur clusters serve as cofactors for several enzymes, electron carrier proteins, and play an important role in mitochondrial iron homeostasis. Mitochondrial iron homeostasis is maintained by the uptake of iron from the cytosol and the incorporation into iron-sulfur clusters and heme groups (reviewed by Levi and Rovida, 2008). In addition, iron is stored in mitochondria. The mitochondrial respiratory chain complexes contain prosthetic groups such as heme (complex II, III, IV and cytochrome c), iron-sulfur clusters (complex I, II, and III), or copper (complex IV).

Ca^{2+} homeostasis

The calcium ion (Ca^{2+}) is an important intracellular messenger that modulates various processes such as muscle contraction, secretion of signalling molecules, respiration, and cell death (reviewed by Rizzuto and Pozzan, 2006; Pizzo and Pozzan, 2007).

Mitochondria are physiological targets and relay points in the intracellular Ca^{2+} signaling due to their close contact with the largest pool of Ca^{2+}, the endoplasmic reticulum (Csordas et al., 2006). It has been shown that mitochondrial membrane protein mitofusin 2 tethers the endoplasmic reticulum to mitochondria enabling the efficient Ca^{2+} uptake (de Brito and Scorrano, 2008). Mitochondrial Ca^{2+} homeostasis is maintained by the uptake and release of Ca^{2+}. A Ca^{2+}-uniporter present in the IMM transports cytosolic Ca^{2+} into mitochondria and an antiporter extrudes Ca^{2+} back to cytosol (Rizzuto and Pozzan, 2006). The uptake of Ca^{2+} by mitochondria activates matrix dehydrogenases of the TCA cycle, thereby stimulating the mitochondrial energy metabolism (Devin and Rigoulet, 2007). Under pathological conditions, an accumulation of Ca^{2+} within mitochondria may lead to the opening of the permeability transition pore ultimately causing cell death.

1.1.3.3 Mitochondria in cell death

Apoptosis

Apoptosis, or programmed cell death, is a coordinated process when aged, damaged, or mutated cells are eliminated by phagocytosis (Hail et al., 2006). Necrosis, so called unprogrammed cell death, is caused by tissue injury, infections, toxins and inflammation. Apoptosis and necrosis are defined by a set of changes in cell morphology. A necrotic cell undergoes swelling, a disruption of the plasma membrane, and cell lysis leading to tissue inflammation. An apoptotic cell undergoes shrinkage, chromatin condensation, DNA fragmentation, and budding of the plasma membrane into so called apoptotic bodies.

Apoptosis plays a central role in embryonic development, morphogenesis, tissue homeostasis, and the immune system (reviewed by Meier et al., 2000). Biochemical features of apoptosis and genes encoding apoptosis have been extensively studied in several model organisms including sponge (*Geodia cydonium*), yeast (*Saccharomyces cerevisiae*), hydra (*Hydra vulgaris*), nematode (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*) and mice (reviewed by Ameisen, 2002; Oberst et al., 2008). Apoptosis can be divided into initiation, effector and degradation phases. Most of the cell's morphological changes occur during the effector phase of apoptosis.

The main players in the effector phase of apoptosis belong to a family of conserved cysteine proteases known as caspases. Since caspases promote the most visible ultrastructural changes characterizing apoptotic cell death, they are assigned as the central executioners of the apoptotic pathway. In addition, other proteases like cathepsins, calpains, serine proteases and the proteasome complex have been implicated as effectors of apoptosis (Hail et al., 2006). Two major signaling pathways can lead to caspase activation: (i) the extrinsic, receptor-mediated pathway and (ii) the intrinsic, mitochondria-mediated pathway (fig. 3) (reviewed by Kroemer et al., 2007).

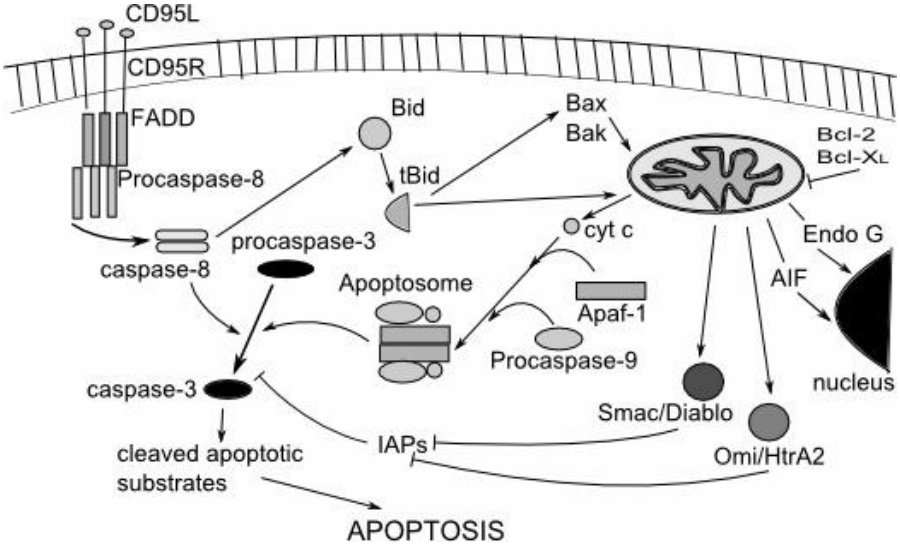

Fig. 3. Receptor- and mitochondria-mediated pathways in mammalian cells. *A family of death receptors e.g. CD95 are responsible for the death receptor-mediated pathway. Binding of CD95 ligands to death receptors forms a death-inducing signaling complex. This complex recruits via the adaptor molecule Fas-associated death domain protein (FADD) and multiple procaspase-8 molecules, resulting in caspase-8 activation. The mitochondria-mediated pathway is responsible for the release of apoptotic proteins from the mitochondrial intermebrane space. Cytochrome c together with the apoptotic protease activation factor-1 (Apaf-1) and procaspase-9 forms the apoptosome that activates effector caspases leading to apoptosis. Apoptosis inducing factor (AIF) and endonuclease G degrade nuclear DNA. A high-temperature requirement (Omi/HtrA2) and a second mitochondria-derived activator of caspases (Smac/Diablo) proteins diminish the inhibitory effect of inhibitor of apoptosis proteins (IAPs) which suppress the caspase cascade. Picture modified from Kaufmann and Hengartner, 2001 and Van Gurp et al., 2003.*

The receptor-mediated pathway involves plasma membrane proteins of the tumour necrotic factor receptor family. Caspase-8 activated through the receptor-mediated pathway cleaves a pro-apoptotic protein, called Bid. The resulting truncated Bid (tBid), in turn, activates the mitochondria-mediated cell death pathway. Consequently, the Bid protein constitutes a link between the receptor- and mitochondria-mediated pathways.

The mitochondrial pathway involves pro-apoptotic members of the Bcl-2 family (e.g. Bid, Bad, Bak, and Bax) (reviewed by Youle and Strasser, 2008). tBid triggers conformational change of cytosolic Bax which oligomerizes and translocates to mitochondria (Lovell et al., 2008). The Bcl-2 family proteins act on the mitochondria and trigger OMM permeabilization possibly by interacting with VDAC and ANT (Zamzami and Kroemer, 2001; Belzacq et al., 2003). Bak, present in the OMM, together with Bax forms a tetrameric channel through which cytochrome c and other mitochondrial apoptotic proteins are released (Wei et al., 2001; Van Gurp et al., 2003). In contrast, several anti-apoptotic members of the Bcl-2 family (e.g. Bcl-2 and Bcl-x_L) block cytochrome c release. Thus, the Bcl-2 family proteins function as regulators of cell death and survival.

Activation of the mitochondrial pathway leads to the release of soluble apoptotic proteins such as cytochrome c, AIF, Smac/Diablo, Omi/HtrA2, and endonuclease G from the mitochondrial intermembrane space to the cytosol (fig. 3). These apoptosis inducing proteins can be divided into two classes: caspase-dependent and caspase-independent (Van Gurp et al., 2003).

AIF causes caspase-independent apoptosis. Overexpression of AIF leads to chromatin condensation, DNA fragmentation, and dissipation of the mitochondrial transmembrane potential (Hail et al., 2006). Endonuclease G, a mitochondrial nuclease, also acts independently of caspases. After release from mitochondria, endonuclease G translocates to the nucleus where it degrades the DNA (Susin et al., 2000).

Cytochrome c triggers apoptosis in a caspase-dependent mode (reviewed by Ow et al., 2008). In the cytosol, cytochrome c induces caspase activation by interacting with Apaf-1 (fig. 3). Activated Apaf-1 undergoes ATP-dependent oligomerization and then binds to procaspase-9. This leads to the formation of the so-called apoptosome, the caspase-9 activation complex that cleaves pro-caspase-3. In turn, caspase-3 triggers the final stage of apoptosis causing characteristic changes in cell morphology. Smac/Diablo and Omi/HtrA2 have similar roles in the apoptotic pathway. Smac/Diablo functions as a caspase-dependent apoptosis inducing protein (Van Gurp et al., 2003). Omi/HtrA2 is involved in both caspase-dependent and caspase-independent cell death. Omi/HtrA2 and Smac/Diablo released from the mitochondrial intermembane space contribute to cell death by antagonizing IAPs (fig. 3). These pro-apoptotic IAP antagonists bind to the baculoviral IAP repeat (BIR) domains of IAPs through their conserved N-terminal IAP binding motifs (IBMs) and thereby release active caspases (Verhagen et al., 2007).

The majority of apoptosis inducing proteins exert additional functions. Evidence demonstrates that apoptosis inducing proteins are also involved in cell metabolism (Hammerman et al., 2004). For example, the pro-apoptotic Bcl-2 family member Bad plays a role in glucose-stimulated insulin secretion in β-cells (Danial et al., 2008). Bax regulates mitochondrial morphology. Bax induces mitochondrial fusion by activating the fusion protein mitofusin 2 (Mfn2) (Karbowski et al., 2006), and participates in apoptotic fragmentation of mitochondria when it colocalizes with the fission protein dynamin-related protein 1 (Drp1) (Karbowski et al., 2002). Cytochrome c participates in the mitochondrial respiration by transferring electrons from complex III to complex IV. AIF is a flavoprotein the absence of which compromises the function of the oxidative phoshporylation by affecting complex I assembly (Vahsen et al., 2004). Muscle- and liver-specific AIF targeted deletion protects from obesity and diabetes (Pospisilik et al., 2007). Omi/HtrA2 functions as a chaperone at normal temperature (Spiess et al., 1999). However, under elevated temperatures or during cellular stress such as heat shock or ischemia, Omi/HtrA2 shows protease activity (Gray et al., 2000). In mice, targeted deletion of Omi/HtrA2 causes mitochondrial dysfunction leading to symptoms characteristic for Parkinson's disease (Martins et al., 2004). Recent human genetic studies, however, do not support the hypothesis that Omi/HtrA2 plays a role in Parkinson's disease (Ross et al., 2008). The cell can undergo several distinct destruction pathways, as the loss of one apoptosis inducing protein can be compensated by other apoptosis inducing proteins. In support of this notion are the knockout models of Smac/Diablo, Omi/HtrA2 or other apoptosis inducing protein that had normal apoptosis (Okada et al., 2002; Martins et al., 2004; Harlin et al., 2001). In summary, these studies suggest that mitochondrial apoptosis inducing proteins, while having a primitive function in cell metabolism, were recruited during evolution for apoptosis. The consequences of apoptosis dysregulation include various mitochondrial disorders and cancer (reviewed by Meier and Vousden, 2007).

The mitochondrial permeability transition

The IMM is normally impermeable to ions and solutes. However, under certain conditions the IMM can become unselectively permeable to molecules less than 1.5 kDa. A high mitochondrial Ca^{2+} concentration can lead to the opening of a large conductance channel, the permeability transition pore (PTP) formed at the contact site between the IMM and OMM. Opening of the PTP causes uncoupling of oxidative phosphorylation with a collapse of the electrochemical proton gradient and abolished ATP production. Moreover, PTP opening is followed by Ca^{2+} release, mitochondrial swelling and release of mitochondrial intermembrane proteins involved in apoptosis (Kroemer et al., 2007).

According to the PTP model, the core of the PTP complex is comprised of the VDAC in the OMM, the ANT in the IMM and cyclophilin D in the matrix. In addition, several proteins have been shown to be associated with the PTP: the peripheral benzodiazepine receptor, hexokinase II, mitochondrial creatine kinase, and pro-apoptotic Bax/Bcl-2 family proteins (Zamzami and Kroemer, 2001). Recent studies from knockout animals indicate that ANT and VDAC are dispensable for the function of mitochondrial PTP (Kokoszka et al., 2005; Baines et al., 2007). However, it is suggested that VDAC and ANT isoforms can substitute each other. For example, ANT1 or VDAC2 are required for Ca^{2+} or tBid induced cell death, respectively (Lee et al., 2009; Roy et al., 2009). In summary, these contradictory findings show that the PTP model requires further investigation. In contrast, it has been shown in mice knockout model that cyclophilin D has an essential role for PTP regulated cell death (Baines et al., 2005). Fibroblasts and hepatocytes from cyclophilin D deficient mice responded to apoptosis inducing proteins like tBid or Bax. However, cyclophylin D deficient fibroblasts and hepatocytes displayed resistance to ROS and Ca^{2+} overload indicating that cyclophilin D-dependent PTP modulates necrotic cell death (Nakagawa et al., 2005). A role of cylophilin D as a potential cancer treatment target is suggested by interaction of cyclophilin D with anti-apoptotic protein Bcl-2 (Eliseev et al., 2009). Altogether, the PTP opening constitutes an essential pathway for apoptotic and necrotic cell death.

1.1.4 Mitochondrial biogenesis

Mitochondrial DNA

Mitochondria contain their own genetic information, the mitochondrial DNA (mtDNA) which is inherited exclusively through the maternal lineage. Human mtDNA is associated with IMM and organized in nucleoprotein complexes, known as nucleoids, which contain 2-8 mtDNA copies (Holt et al., 2007). The copy number of mtDNA per cell differs depending on the cell type. For example, a human spermatozoa and a mature oocyte have 10^2 and 10^5 copies of mtDNA, respectively.

The mitochondrial genetic code is different from the universal genetic code. The mitochondrial genetic code of vertebrates and invertebrates differs from the universal genetic code in four of the 64 codons, while the mitochondrial genetic code of yeast varies from the universal genetic code in three of the 64 codons. One common feature of the

mitochondrial genome of vertebrates, invertebrates and yeast is the TGA codon that is used for tryptophan instead of a stop codon. In addition to the mitochondrial genetic code differences, the size of the mitochondrial genome also varies across the species. In plants, the mitochondrial genome consists of more than $200x10^3$ base pairs, whereas, the human mitochondrial genome is 10 times smaller and consists of 16,569 base pairs (Scheffler, 2001).

The human mitochondrial genome is a circular double-stranded molecule consisting of a heavy strand (H) and a light strand (L). It encodes 22 transfer RNAs, 2 ribosomal RNAs, and 13 subunits of the mitochondrial respiratory chain complex and is devoid of introns and has only one non-coding region that includes a displacement loop (D-loop). The D-loop contains control elements for mtDNA transcription and replication: the H and L strand promoters, and the mtDNA H-strand replication origin. Transcription from the H and L strand promoters generates the polycistronic precursor RNAs that are further processed to yield the individual transfer RNAs, ribosomal RNAs, and messenger RNAs (mRNA). Most proteins involved in mammalian mtDNA replication and transcription are constituents of nucleoids (Wang and Bogenhagen, 2006). The importance of mtDNA maintainance proteins are emphasized in mitochondrial diseases associated with mtDNA mutations (reviewed by Tyynismaa and Suomalainen, 2009). The mtDNA mutations are proposed to be a major factor in cellular aging and neurodegenerative diseases (Schapira, 2002; Kraytsberg et al., 2006).

Mitochondrial protein import

Nucleus-encoded mitochondrial proteins are synthesized as precursor proteins in the cytosol and translocated into mitochondria. The majority of precursor proteins targeted to the matrix, the intermembrane space and the inner membrane carry N-terminal positively charged presequences capable of forming basic amphipathic α-helices. In contrast, most outer membrane proteins and carrier proteins of the inner membrane harbor targeting information within their mature sequences (reviewed by Gakh et al., 2002). The correct import, membrane translocation and sorting of mitochondrial proteins to different mitochondrial subcompartments are ensured by complex protein-import machineries spanning the OMM and IMM (reviewed by Baker et al., 2007; Chacinska et al., 2009).

All precursor proteins are imported into mitochondria through the universal OMM translocase, the TOM complex. The main components of the TOM complex include the receptors Tom20 and Tom70, and the translocation channel Tom40. Tom20 is responsible for the targeting signal recognition, while Tom70 docks the hydrophobic precursor proteins (Saitoh et al., 2007). The Tom40 subunit acts as a membrane translocase (Model et al., 2008). The translocation pathway through the IMM diverges to sort the precursor proteins to their destined mitochondrial subcompartments.

Translocation of soluble matrix or inner membrane proteins containing N-terminal targeting signals across the IMM requires the TIM23 complex (Popov-Celeketic et al., 2008). In contrast, the TIM22 complex is involved in the import of inner membrane proteins with internal signal sequences (Peixoto et al., 2007). The import of proteins through the IMM is driven by the electrochemical potential ($\Delta\psi$) and the chaperone

mtHsp70 (Young et al., 2003). Precursor proteins targeted to various mitochondrial subcompartments are further proteolytically processed by peptidases.

The mitochondrial processing peptidase cleaves matrix-targeting presequences, while the intermembrane space-sorting signals are removed by the inner membrane peptidase (Gakh et al., 2002). The mitochondrial intermediate peptidase performs a second cleavage of proteins that were initially processed by the mitochondrial processing peptidase. Many precursor proteins destined to the mitochondrial matrix or the inner membrane are processed in two steps by the mitochondrial processing peptidase and the mitochondrial intermediate peptidase. An inefficient processing of precursor proteins can cause mitochondrial diseases because the targeting signal might interfere with protein folding or further sorting. The mitochondrial protein-import machinery guarantees a continuous delivery of proteins required for mitochondrial growth and division.

Mitochondrial fusion and fission

Mitochondria are highly dynamic organelles, the morphology of which can be regulated by fusion and fission. Mitochondria undergo fusion and fission in response to different biological signals (reviewed by Detmer and Chan, 2007). Fusion and fission allow the mixing of metabolites and mtDNA required for the proliferation and distribution of mitochondria (Legros et al., 2004). According to a recent transient fusion model, the length of fusion events was sufficient to exchange soluble intermembrane space and matrix proteins, proposing that even transient fusion enhances functional stability of mitochondria (Liu et al., 2009).

Mitochondrial fusion involves both OMM and IMM components (Detmer and Chan, 2007). The OMM fusion is controlled by an evolutionary conserved large GTPase: Fzo1p in yeast, and its mammalian homologs, mitofusins 1 and 2 (MFN1 and MFN2) (Chen et al., 2003). The IMM fusion requires a dynamin-like GTPase: Mgm1 in yeast and its mammalian homolog OPA1 (Frezza et al., 2006).

Mitochondrial fission requires a dynamin-related GTPase, Drp1 (yeast homolog Dnm1p) (Smirnova et al., 2001) and a small protein hFis1 (yeast homolog Fis1) (James et al., 2003). Drp1 from the cytoplasm is translocated to mitochondria where it binds to hFis1 and contributes to constriction of the OMM.

In addition to maintaining mitochondrial morphology, fission and fusion proteins modulate cell death. The mitochondrial fission machinery is responsible for mitochondrial fragmentation during apoptosis. Inhibition of Drp1 and Fis1 prevents mitochondrial fragmentation and delays apoptosis (Lee et al., 2004; Estaquier and Arnoult, 2007). In contrast, mitochondrial fusion plays a protective role in apoptosis. Apoptosis is prevented by overexpression of mitofusins (Sugioka et al., 2004). OPA1 regulates apoptosis by controlling crista remodeling and cytochrome c redistribution (Frezza et al., 2006). However, contradictory results suggest that cytochrome c is released without cristae junctions widening (Sun et al., 2007; Yamaguchi et al., 2008). Yamaguchi proposed a model where tBit treatment opens cristae junctions by disassembling OPA1 oligomers (Yamaguchi et al., 2008), disrupts cardiolipin-cytochrome c association (Kim et al., 2004) and releases cytochrome c through Bax/Bak pores (Lovell et al., 2008). Thus,

mitochondrial morphology depends on the balance between antagonistic fusion and fission processes.

1.1.5 Mitochondria in disease

Disturbances in the mitochondrial function can lead to characteristic metabolic and neurodegenerative disorders. A large number of factors contribute to metabolic and neurodegenerative mitochondrial disorders, including genetic factors, oxidative stress, aging, reduced oxidative phosphorylation, β-oxidation, and altered ion homeostasis (reviewed by Wallace, 2005).

Obesity and diabetes

Obesity is a worldwide metabolic disorder associated with type 2 diabetes and cardiovascular diseases (reviewed by Kim et al., 2008). Obesity increases the risk to develop insulin resistance. Insulin resistance is defined as a decreased response by skeletal muscle, liver and adipose tissue to insulin. Consequently, insulin resistance leads to the metabolic disorder, type 2 diabetes (reviewed by Kahn et al., 2006; Muoio and Newgard, 2008).

Glucose homeostasis is maintained by insulin-responsive organs: skeletal muscle, liver and adipose tissue. Insulin secreted by pancreatic β-cells decreases glucose production from liver, and increases glucose uptake, storage and utilization in skeletal muscle and adipose tissue. Oxidative mitochondrial metabolism plays a central role in the glucose-stimulated insulin secretion pathway from pancreatic β-cells. Glucose oxidation promotes the insulin secretion through an increased ATP production. An increased ratio of ATP to ADP causes the closure of ATP-sensitive K^+ channels, depolarization of the plasma membrane, and the opening of voltage-sensitive Ca^{2+} channels. The resulting increase in the cytosolic Ca^{2+} concentration triggers insulin secretion (Kahn et al., 2006). In addition, a role for mitochondrial uncoupling protein, UPC2, in insulin secretion has been suggested in mice models used for studying obesity and obesity-related diabetes (Zhang et al., 2001; Joseph et al., 2004). These studies indicate that an increased expression of UCP2 and a UCP2-mediated proton leak decreases ATP production. A decreased ATP production impairs insulin secretion, resulting in β-cell dysfunction and metabolic disturbances. However, recent studies show that overexpressed UCP3 or carnitine palmitoyltransferase-1, which is involved in long chain fatty acyl CoA transport to mitochondria (reviewed by Kerner and Hoppel, 2000), have a protective role in insulin resistance (Choi et al., 2007; Bruce et al., 2009).

According to one model, defects in glucose transport and lipid metabolism impair the insulin signaling pathway. The insulin signaling cascade is inhibited by fatty acid metabolites. Fatty acid metabolites deriving from adipocytes are elevated by stress or excess nutrient intake (Bergman and Ader, 2000). Accumulation of fatty acid and lipid metabolites such as fatty acyl-CoAs, diacylglycerol, and ceramide, activates serine/threonine kinases that phosphorylate the insulin receptor substrate family members

(Itani et al., 2002; Bandyopadhya et al., 2006; Holland et al, 2007). Phosphorylated insulin receptors cause increased glucose production in liver, reduced glucose uptake in the skeletal muscle and finally insulin resistance (fig. 4) (Lowell and Shulman, 2005). Whereas, alternative obesity studies suggest that increased rates of fatty acid oxidation and mitochondrial overload of lipids contribute to insulin resitance (Koves et al., 2008).

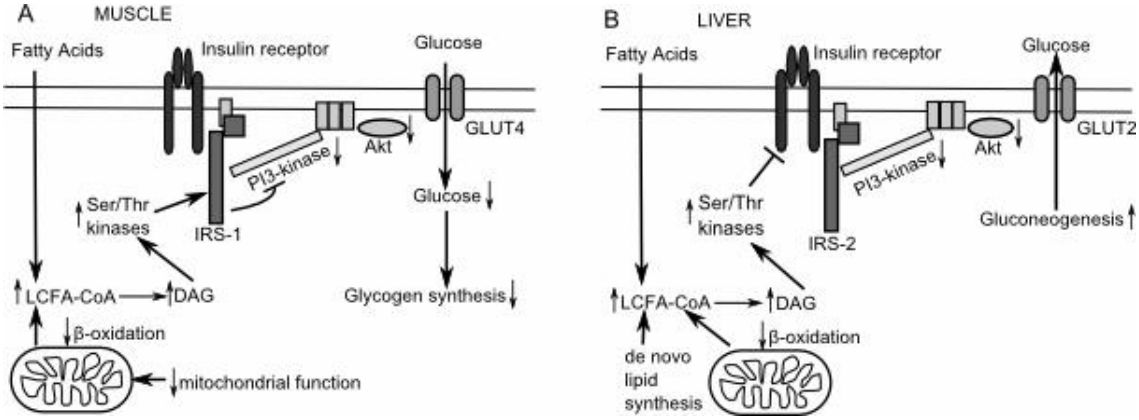

Fig. 4. The molecular mechanism of insulin resistance in (A) skeletal muscle and (B) liver. *(A) Intracellular long-chain fatty acyl CoA (LCFA-CoA) and diacylglycerol (DAG) levels are increased due to the increased delivery from plasma or from decreased mitochondrial β-oxidation, caused by mitochondrial dysfunction. Increased levels of LCFA-CoA and DAG activate serine/threonine kinases such as protein kinase C. The activated kinases phosphorylate the serine residue of the insulin receptor substrate-1 (IRS-1) and inhibit insulin-induced phosphatidylinositol 3-kinase (PI3K). Inhibition of PI3K results in a reduction of insulin-stimulated protein kinase B (Akt) activity. Decreased Akt activity results in suppression of insulin-stimulated glucose transport through GLU4 transporter. Consequently, this leads to a reduced insulin-induced glucose uptake and glycogen synthesis. (B) Serine/threonine kinases are activated by an increased level of DAG due to an increased delivery of fatty acids from plasma or an increased de novo lipid synthesis, or from impaired β-oxidation. Activated serine/threonine kinases reduce insulin receptor kinase activity and impair IRS-2 tyrosine phosphorylation, leading to a reduced Akt activity and increased gluconeogenesis. Picture modified from Morino et al., 2006.*

In obesity and type 2 diabetes, the increased release of hormones such as leptin and adiponectin from adipose tissue is associated with insulin resistance (Scherer, 2006). Moreover, in obese individuals an increased release of the proinflammatory cytokines tumour necrosis factor-α, interleukin-6, and C-reactive protein might lead to inflammation and insulin resistance (reviewed by Schenk et al., 2008).

Several genes associated with obesity and diabetes have been identified (O'Rahilly and Farooqi, 2006; Barrosos, 2005). Leptin is the most extensively investigated obesity gene. Leptin acts through the central nervous system to regulate food intake and energy expenditure (Friedman and Halaas, 1998). Genome-wide association studies have identified novel genetic variants that could contribute to the development of type 2 diabetes. Type 2 diabetes-associated variants are located near genes such as cyclin-depended kinase 5, fat mass and obesity associated protein, and insulin-like growth factor 2 binding protein 2 (Zeggini et al., 2007; Scott et al., 2007). Recently several studies of gene networks have identified candidate genes for obesity, including zinc finger protein 90,

lipoprotein lipase (Lpl), protein phospatase 1-like (Ppm1l), and LACTB (Schadt et al., 2005; Chen et al., 2008). *Lpl*, *Ppm1l* and *Lactb* were validated as obesity genes in mice knockout and transgenic models, respectively. Besides, environmental factors such as an increased energy intake and decreased energy expenditure influence the development of obesity (Kahn et al., 2006).

Oxidative stress and aging

Oxidative stress is defined as an imbalance between the production and the elimination of ROS. Mitochondria are both targets and sources of ROS. Complex I and complex III of the mitochondrial respiratory chain are the main production points of ROS. Accumulation of ROS can induce opening of the mitochondrial PTP (Lemasters et al., 1998). Cell survival is dependent on the antioxidant system that includes enzymatic scavengers: superoxide dismutase, catalase, and glutathione peroxidase. Thus, the reduced efficiency of the antioxidant system leads to ROS accumulation (Melov et al., 1999).

Excessive ROS can promote oxidative damage to membrane lipids, proteins and DNA (Barja, 2004). The mitochondrial theory of aging postulates that the mtDNA is located in close proximity to the main source of ROS production. ROS damage to mtDNA impairs the mitochondrial respiratory chain function, which further enhances ROS generation. In turn, enhanced ROS generation leads to more mtDNA damage creating a vicious cycle (reviewed by Beckman and Ames, 1998; Finkel and Holbrook, 2000). Recently, this theory was challenged by studies on long-lived vertebrate species, mutants and transgenic animals (reviewed by Bufferenstein et al., 2008). Nevertheless, many lines of evidence demonstrate that an age-dependent increase in oxidative stress and mitochondria dysfunctions contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (reviewed by Lin and Beal, 2006).

Neurodegenerative diseases

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by a selective loss of neurons in the hippocampus and the cerebral cortex (Lin and Beal, 2006). The pathological features of AD are extracellular amyloid plaques that contain cleavage products (amyloid-β (Aβ)) of amyloid precursor protein and intracellular neurofibrillary tangles components of hyperphosphorylated tau protein. Mutations in three genes encoding the amyloid precursor protein, presenilin 1 and 2 have been linked to AD. It was shown that Aβ inhibits respiration and enzyme activity of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase in isolated mitochondria (Casley et al., 2002). Aβ can directly induce oxidative stress and neuronal death through the interaction with mitochondrial alcohol dehydrogenase (Lustbader et al., 2004) and cyclophilin D (Du et al., 2008). The amyloid precursor protein causes mitochondrial fragmentation in neurons by modulating fusion and fusion proteins (Wang et al., 2008). These observations support a link between mitochondria and AD.

Parkinson's disease (PD) is the most common neurodegenerative movement disorder (Lin and Beal, 2006). Selective loss of dopaminergic neurons in the substantial nigra and

the appearance of cytoplasmic α-synuclein-immunoreactive inclusions, termed Lewy bodies, are the symptoms of PD. Mutations linked to parkinsonism include α-synuclein, parkin, DJ-1 and PINK1 genes: α-synuclein can polymerize into filaments and is found in Lewy bodies (Fornai et al., 2005); parkin encodes a ubiquitine E3 ligase that targets ubiquitine labeled substrates for degradation by the ubiquitin-proteasome pathway (Kitada et al., 1998) and is involved in mitochondrial autophagy (Narendra et al., 2008); DJ-1 participates in the oxidative stress response (Bonifati et al., 2003); PINK1 maintains mitochondrial membrane potential and protects from apoptosis induced by proteasome inhibitors (Valente et al., 2004). Some of these gene products contribute to mitochondrial dysfunction. For example, mutated parkin and proteasome inhibition results in mitochondrial swelling, cytochrome c release and apoptosis (Darios et al., 2003), parkin and PINK1 deficiency leads to mitochondrial fragmentation (Lutz et al., 2009), while overexpression of α-synuclein impairs mitochondrial activity and increases oxidative stress (Hsu et al., 2000).

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease. HD is characterized by cognitive impairment and loss of neurons in the cortex and the striatum. HD is caused by multiple CAG repeat expansion in the huntingtin gene. The toxic effect of polyQ expansion degenerates neurons in the striatum and the cerebral cortex (Lin and Beal, 2006). Brain mitochondria isolated from transgenic mice which express full-length huntingtin containing 72 CAG repeats show an impaired Ca^{2+} uptake and an increased susceptibility to the mitochondrial PTP activation (Panov et al., 2005). Mutant huntingtin causes mitochondrial fragmentation that is likely mediated through inhibition of mitochondrial fusion (Wang et al., 2009). Furthermore, the mitochondrial serine protease Omi/HtrA2 is involved in HD. Mouse mnd2 (motor neuron degeneration 2) phenotype has a mutation in Omi/HtrA2 and show common features of HD, including involuntary movements and massive loss of striatal neurons (Rathke-Hartlieb et al., 2002).

Amyotrophic lateral sclerosis (ALS) leads to progressive muscle wasting and weakness. 90% of ALS cases are sporadic, while 10% of ALS cases are familial. 20% of familial ALS are caused by mutations in Cu/Zn superoxide dismutase (SOD). Mutant SOD forms aggregates in the intermembrane space and the matrix of brain mitochondria triggering mitochondrial dysfunction (Vijayvergiya et al, 2005; Magrane et al., 2009). However, also during normal aging SOD aggregates in motor neurons, aggregates of Aβ in AD and α-synuclein in dopaminergic neurons result from the accumulation of misfolded and damaged proteins (Lin and Beal, 2006). Such protein aggregates might arise as a consequence of oxidative damage, mitochondrial dysfunction and impaired proteosome function (Glickman and Ciechanover, 2002).

Mutations in mitochondrial proteins can also cause neurodegenerative diseases (reviewed by Knott et al., 2008). For example, autosomal dominant optic atrophy is characterized by degeneration of the optic nerve, retinal ganglion cells, and progressive loss of vision (Delettre et al., 2002). Mutations in the mitochondrial fission protein OPA1 cause autosomal dominant optic atrophy. Down regulation of OPA1 by RNAi causes mitochondrial fragmentation and leads to apoptosis which partially explains the disease mechanism (Lee et al., 2004).

Based on a large number of studies, it is established that neurodegenerative diseases involve mitochondria as a final common pathway. Thus, therapies targeting processes such

as ROS generation, the proteosome and mitochondrial proteins involved in diseases could provide a treatment for neurodegenerative diseases.

1.2 Penicillin binding proteins and β-lactamases

1.2.1 Penicillin-binding proteins

Penicillin-binding proteins (PBPs) form a distinct family of acyl serine transferases. PBPs catalyze the final steps in the synthesis of the major bacterial cell wall component, peptidoglycan (PG) (reviewed by Sauvage et al., 2008).

In gram-positive bacteria, PG constitutes a thick multilayer outside the bacterial cytoplasmic membrane. In contrast, gram-negative bacteria have a thin layer of PG located in the periplasmic space between outer and inner bacterial membranes. PG confers a mechanic and osmotic resistance to the bacterial cell wall. In addition, PG dictates cell shape since it undergoes constant chemical changes during cell growth and division (reviewed by Macheboeuf et al., 2006). During growth and division, bacteria can synthesize a new PG and hydrolyse the existing PG in order to enable an insertion of new material (Scheffers and Pinho, 2005). The synthesis and hydrolysis of PG require a coordination of several different enzymes. It was proposed and subsequently demonstrated by protein-protein interaction studies (von Rechenberg et al., 1996) that a multienzyme complex participates in the PG synthesizing machinery (fig. 5). The PG synthesizing machinery combines activities of PG hydrolases and PG synthases such as transpeptidases and transglycosylases.

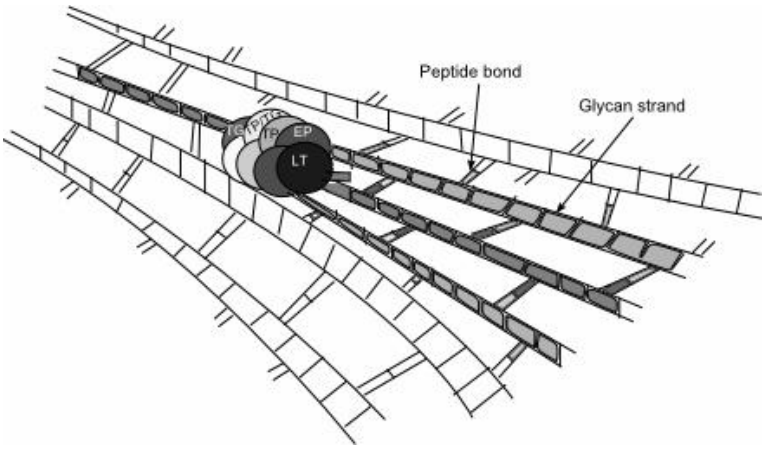

Fig. 5. Proposed model of peptidoglycan growth*. A multienzyme complex synthesizes three new cross-linked glycan strands (grey) with the assistance of enzymes such as transpeptidase (TP), bifunctional transpeptidase-transglycosylase (TP/TG) and transglycosylase (TG). A multienzyme complex attaches these glycan strands to the cross bridges on both sides of a docking strand (single grey strand) and concomitantly degrades this strand. Enzymes such as lytic transglycosylase (LT) and endopeptidase (ED) participate in the hydrolysis process. Picture modified from Höltje, 1998.*

The assembly of PG requires the polymerization of glycan chains composed of N-acetylglucosoamine (NAG) and N-acetylmuramic acids (NAM). Subsequently, these glycan chains are cross-linked via D-alanyl-D-alanine bond of muramyl pentapeptide units (fig. 6) (Ghuysen, 1991; Macheboeuf et al., 2006).

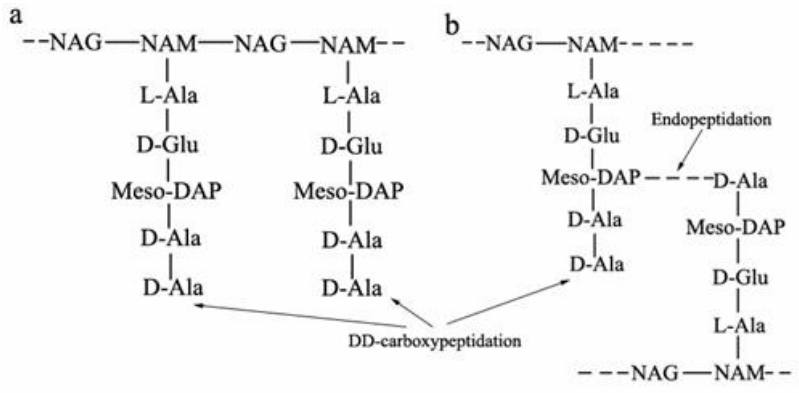

Fig. 6. Chemistry of the peptidoglycan synthesis. *The basic unit of the PG is a disaccharide-pentapeptide composed of amino sugars NAG and acid NAM linked together by β-1,4 glycosidic bonds. PBP catalyze the (a) transglycosylation and (b) transpeptidation. The transglycosylation reaction involves formation of the glycosidic bonds of the PG, whereas the peptide side-chains of different PG strands are cross-linked by transpeptidase activity. DD-carboxypeptidases remove terminal D-alanine from the monomeric or dimeric disaccharide pentapeptide side-chains and DD-endopeptidases cleave cross-linked peptide side-chains (Höltje, 1998). Meso-DAP (meso-diaminopimelic acid) in gram-negative bacteria is changed to L-Lys in gram-positive bacteria.*

All PBPs harbor three conserved amino acid motifs: SXXK, (S/Y)XN and (K/H)(T/S)G (Ghuysen, 1991; Massova and Mobashery, 1998). The SXXK motif contains the active site serine residue that underoges acylation and deacylation cycles, and forms a covalent bond with substrate. The (S/Y)X(N/T) motif points to the active site cleft and participates in the hydrolysis. The (K/H)(T/S)G motif is involved in substrate docking.

PBPs are grouped into two classes: the high and low molecular weight PBPs (Ghuysen, 1991; Sauvage et al., 2008). The high molecular weight PBPs are bifunctional enzymes catalyzing both the transpeptidation of the peptide chains and the transglycosylation of the glycan strand. In contrast, the low molecular weight PBPs act as DD-carboxypeptidases hydrolysing the D-alanine residue of the pentapeptide. High and low molecular weight PBPs are further subdivided into classes A, B, and C on the basis of amino acid sequence similarities.

PBPs from different classes perform different functions associated with bacterial cell shape and division machineries. *E. coli* PBP2 and PBP5 are involved in bacterial cell shape maintenance (Nanninga, 1998; Ghosh et al., 2008), whereas, *Streptococcus* PBP3 and *Staphylococcus* PBP1 are implicated in bacterial cell division (Morlot et al., 2004; Pereira et al., 2009).

PBPs are target sites of penicillins and other β-lactam antibiotics. β-lactam antibiotics are compounds produced by fungi and bacteria that act by inhibiting bacterial cell wall synthesis. β-lactam antibiotics acylate the active-site serine residue of PBPs thus rendering the PBP devoid of transpeptidase activity (Ghuysen, 1991).

1.2.2 β-lactamases

β-lactamases are enzymes responsible for bacterial resistance to β-lactam antibiotics (e.g. penicillins and cephalosporins). β-lactamases inactivate β-lactam antibiotics by hydrolyzing the peptide bond of the β-lactam ring rendering the antibiotic inactive (fig. 7). During evolution bacteria producing β-lactamases gained a distinct survival advantage. However, resistance to β-lactam antibiotics is an increasing clinical problem nowadays. Infections caused by resistant bacterial strains can lead to epidemics and greater risk of death.

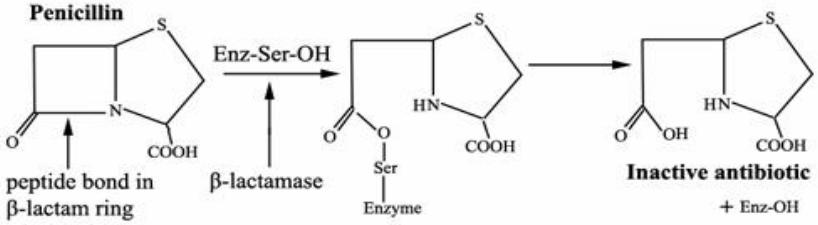

Fig. 7. Penicillin inactivation reaction. *The reactive site of penicillin is the peptide bond of its β-lactam ring. Inactive penicillin is formed from the hydrolysis of the β-lactam ring.*

β-lactamases are categorized into four classes, A, B, C and D based on the amino acid sequence similarity and the catalytic mechanism (Bush et al., 1995). Classes A, C, and D are active-site serine enzymes, whereas class B, termed metallo β-lactamases, requires zinc ions (Massova and Mobashery, 1998).

Serine β-lactamases share structural similarities with the targets of β-lactam antibiotics, the PBPs. Based on the published studies it has been accepted that serine β-lactamases and PBPs emerged from the same ancestral enzyme (Ghuysen, 1994; Medeiros, 1997). Structure-based phylogeny of the serine β-lactamases and PBPs indicate that the class C serine β-lactamases are more similar to low molecular weight PBPs (Massova and Mobashery, 1998). Furthermore, the class C serine β-lactamases predates the divergence of classes A and D from the common ancestor (Hall and Barllow, 2003). X-ray crystallographic comparison of several PBPs and the class C β-lactamases revealed structural similarities such as a single domain containing two regions, one α/β and one all-helical with the catalytic site lying between them, and the three conserved amino acid motifs described in chapter 1.2.1 (fig. 8).

Thus, PBPs and β-lactamases constitute a large superfamily of active-site serine enzymes that are responsible for the synthesis and maintenance of PG.

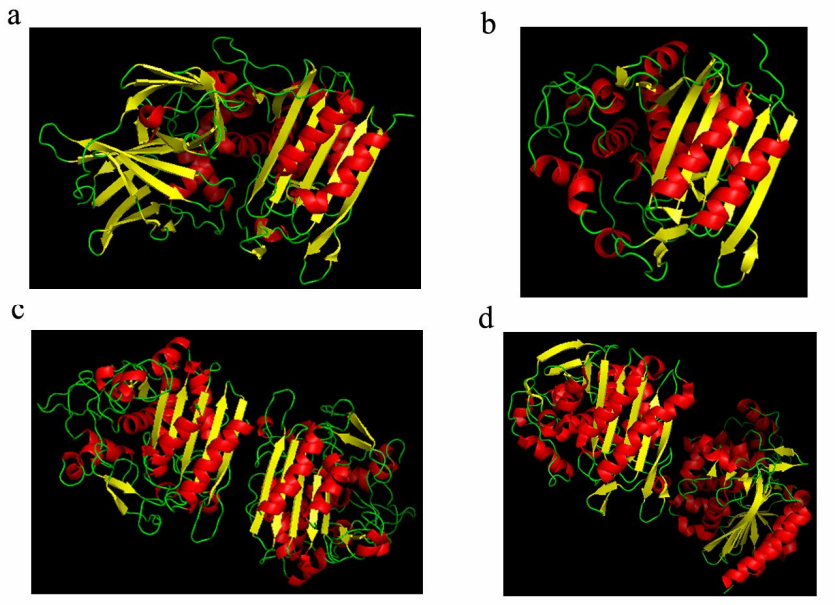

Fig. 8. *Crystal structures of (a) D-aminopeptidase from Ochrobactum anthropii (Bompard-Gilles et al., 2000), (b) the DD-carboxypeptidase from Streptomyces R61 (Kelly and Kuzin, 1995), (c) the class C β-lactamase from Burkholderia gladioli (Wagner et al., 2002), (d) the class C β-lactamase from Enterobacter cloacae P99 (Lobkovsky et al., 1993). 3-dimensional (3D) models were made with the program Pymol.*

1.3 The active-site serine protein LACTB

LACTB is a homolog to bacterial PBP and β-lactamase enzymes. LACTB has not yet been systematically analyzed and only some scattered information is available about its physiological function. LACTB was mentioned in the literature for the first time when the cDNA encoding LACTB was cloned from mouse liver by Smith in 2001 (Smith et al., 2001). Mouse LACTB is a 551 amino acid long containing an active site motif related to class C β-lactamases (Rawlings and Barrett, 1994). Homologues of LACTB were identified in several mammals, toad, fish, and nematodes, but not in yeast or fly.

LACTB has been detected in mitochondrial proteome studies soon after the first report of LACTB. LACTB has been identified in the mitochondria of human and mouse heart, mouse brain, liver, kidney and other tissues suggesting that LACTB is a ubiquitous mitochondrial protein (Taylor et al., 2003; Mootha et al., 2003; Gaucher et al., 2003; Pagliarini et al., 2008). It has been reported that LACTB, also referred in literature as MRP-L56, was co-purified with the 39S subunit of the mammalian mitochondrial ribosome (Koc et al., 2001). LACTB was also identified in the extracellular space and associated with membrane fraction indicating that LACTB might be localized in other cellular compartments (Clark et al., 2003; Zhang et al., 2008).

Several studies revealed that LACTB is regulated at both the transcriptional and the posttranslational levels. The LACTB mRNA expression was increased by insulin in human

skeletal muscle suggesting that LACTB might play a role in anabolic processes (Rome et al., 2003). LACTB is regulated at the posttranslational level by acetylation and phosphorylation. Lysine acetylation of LACTB occurs during starvation indicating that LACTB is modulated by acetyltransferases and deacetylase enzymes (Kim et al., 2006; Schwer et al., 2006). Conversely, the first serine residue located in the catalytic -SISK- motif of LACTB was phosphorylated under normal conditions suggesting that LACTB is activated by a specific phosphoprotein phosphatase (Lee et al., 2007). Altogether, these studies indicate that the LACTB function might be regulated by posttranslational modifications.

Microarray analysis of murine macrophages after infection with virulent intracellular bacteria, *Brucella melitensis,* showed that *Lactb* was down regulated. *Lactb* grouped together with down-regulated macrophage genes involved in cell growth and metabolism (He et al., 2006). In contrast, microarray analysis of HeLa carcinoma cells repressed by bovine papillomavirus E2 protein revealed that *Lactb* was induced in the senescence undergoing HeLa cells (Johung et al., 2007). Furthermore, a PBP homolog *Lact-1* was up regulated in *C. elegans* after infection with fungus, *Drechmeria coniospora* (Pujol et al., 2008). These findings propose that LACTB might also have a function in immune response against viral or fungal infections.

Conclusive evidence for the role of LACTB in metabolism came when *Lactb* was validated to be a causal gene for obesity in gene network studies (Schadt et al., 2005; Chen et al., 2008). In *Lactb* transgenic mice, the fat mass to lean mass was increased by 20% compare to wild-type controls (Chen et al., 2008). Further characterization of *Lactb* transgenic mice revealed that LACTB is associated with fatty acid metabolism (Yang et al., 2009). Liver gene expression profiling of *Lactb* transgenic mice showed down regulation of fatty acid metabolic pathway. Interestingly, in genome-wide cDNA expression screen *Lactb* was identified as a repressor gene implicated in sterol regulatory element-binding protein pathway (Chatterjee et al., 2009). Sterol regulatory element-binding proteins, a family of transcription factors, regulate the expression of genes involved the synthesis of cholesterol, fatty acids and triglycerides.

At the time this project was started neither enzymatic substrates nor physiological function of mammalian LACTB were known.

2. AIMS OF THE STUDY

The goal of this study was to investigate LACTB protein in order to elucidate its physiological role.

The specific aims of this study were the following:

- To clone the *Lactb* gene for expression of LACTB protein in *E. coli* and mammalian cells for cellular biology studies.
- To investigate the expression level of LACTB mRNA.
- To purify the recombinant LACTB protein from *E. coli* and endogenous LACTB from rat liver mitochondria for biochemical and structural characterization.
- To identify protein-protein interaction partners of LACTB.
- To perform a phylogenetic analysis to investigate the evolutionary history of LACTB.
- To perform homology modeling to generate a structural model of LACTB.

3. MATERIALS AND METHODS

The methods used in this study are shortly described together with some methodological considerations. Further details about methods and materials are available in the original publications.

3.1 Bioinformatics (II, III)

Genomic DNA, EST, and protein data bases comprised of eukaryotic genome projects were searched for LACTB homologs using BLAST program. The mitochondrial targeting sequence was predicted using Mitoprot program. Binary amino acid alignments were made using DOTLET and LALIGN. Multiple alignments of amino acid sequences were made using ClustalW. Phylogenetic analysis was performed by maximum likelihood using the PROMLK program of the Phylip package. 3D structure predictions performed using the I-TASSER server and visualized using PyMOL.

3.2 Molecular Biology (I, III, IV)

Polymerase chain reaction

A full-length LACTB cDNA from mouse *Mus musculus* (clone BC046293: pCMV SPORT6.1-mLACTB) was amplified by the polymerase chain reaction (PCR) using Phusion DNA polymerase (Finnzymes). Primers used for the PCR are specified in the original paper **I.**

Plasmid generation. Molecular cloning

Cloning and high-level expression of tagged recombinant protein in *E. coli* or mammalian cells were performed according to the Invitrogen Instruction manual using Gateway technology (Invitrogen).
Plasmids, bacterial strains and cell lines. Gateway entry and destination plasmids used for prokaryotic expression system or for eukaryotic expression system are summarized in table 1. HeLa cells expressing red fluorescent protein targeted to mitochondria (mtRFP) were used for recombinant LACTB protein expression. Chemically competent *E. coli* used for recombinant LACTB protein expression in the prokaryotic expression system are described in table 2. Expression of recombinant protein is induced by isopropyl-β-D-thiogalactopyranoside (IPTG) in *E. coli* strains expressing the T7 RNA polymerase.

Table 1. *Gateway plasmids used in the experiments.*

Entry plasmid	
pENTR/SD/D-TOPO	
Destination plasmid	**Fusion tag**
pDEST 15	N-terminal GST
pDEST 17	N-terminal His
pDEST 24	C-terminal GST
pET-DEST 42	C-terminal His
pcDNA-DEST 47	C-terminal GFP

Table 2. *E. coli strains used in experiments.*

E. coli strain	***Application***
DB3.1	Gateway plasmids contain the toxic ccdB gene. *E. coli* strain DB3.1 was used to propagate Gateway plasmids without insert.
TOP10	*E. coli* strain TOP10 alows stable replication of high-copy number plasmids and was used to propagate pENTR/SD/D-TOPO entry plasmid with insert.
XL2-blue	*E. coli* strain XL2-blue allows high-efficiency cloning and was used to obtain a high-quality plasmid DNA.
BL21 (DE3)	*E. coli* strain BL21 (DE3) is designed for use with any bacteriophage T7 promoter based expression systems. It was used for high-level expression of recombinant protein in E. coli.

Gateway cloning reaction. The Gateway cloning reaction was carried out to insert the PCR product into the plasmid. Briefly, fresh PCR product (50-100 ng/µl) purified from 0.8% agarose-gel using the NucleoSpin Extract Kit (Macherey-Nagel) was mixed with pENTR/SD/D-TOPO entry plasmid. *E. coli* strain TOP10 were transformed with this plasmid DNA by heat shock treatment and thereafter plated onto agar plates containing appropriate antibiotics. After incubation overnight at +37°C, single colonies were picked and used to inoculate 5 ml Luria Bertani (LB) medium containing appropriate antibiotics. The pENTR/SD/D-TOPO plasmid DNA was purified using the NucleoSpin kit (Macherey-Nagel) following the manufacturer's instructions.

Gateway recombination reaction. The Gateway recombination reaction was performed between the pENTR/SD/D-TOPO plasmid containing the mouse LACTB (mLACTB) insert and five different destination plasmids. The Gateway recombination reaction yielded five expression plasmids (fig. 9). *E. coli* strain XL2-blue was transformed with the recombination reaction mixture. Expression plasmid DNA was purified as mentioned above. *E. coli* strain BL21 (DE3) was transformed with this expression plasmid for prokaryotic expression systems.

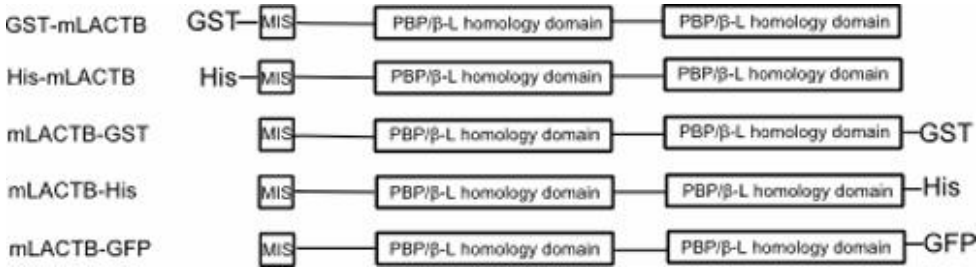

Fig. 9. Schematic representation of mLACTB expression plasmids used in the experiments. *mLACTB fused to N- or C-terminal GST or His tags were used to investigate the recombinant mLACTB expression in E. coli. Full-length mLACTB C-terminally fused to GFP was generated for expression in mammalian cells. MIS-mitochondrial import sequence.*

Restriction endonuclease DNA digestion and sequencing

Restriction endonuclease DNA digestion was performed to verify positive plasmids containing the mLACTB insert (New England Biolab's manual instructions). Plasmids verified by restriction endonuclease DNA digestion were sent for sequencing at the sequencing facility of Helsinki University. DNA sequencing was carried out to verify that the mLACTB insert had a correct reading frame.

Expression and purification of recombinant mLACTB

E. coli strain BL21 (DE3) transformed with expression plasmids was cultured in LB medium containing appropriate antibiotic. Further protein expression and purification procedures are described in the original paper **I**.

Analysis of LACTB mRNA expression

The Human Multiple Tissue Expression array (BD Biosciences, Clontech) was used for analysis of LACTB mRNA expression. Detailed method of sample preparation and hybridization is described in the original paper **IV**.

3.3 Biochemistry (III)

Experimental animals

Wistar rats were kept in an animal facility and treated according to the ethics guidelines of the European Union.

Liver preparations of ethanol-treated mice

Ethanol-treated liver samples were a kind gift from Kai Lindros. Mice had been fed with ethanol or with a control diet for six weeks. Livers were excised and directly frozen in liquid nitrogen. Frozen liver was ground to powder and dissolved in SDS-PAGE sample buffer for LACTB protein analysis.

Mitochondria preparations

Isolation of mitochondria. Mitochondria were isolated from liver, kidney, lung, skeletal muscle, heart, brain, spleen, testis, lacrimal gland, adrenal gland, thymus and brown fat tissues of male Wistar rats as described in the original paper **III**. Mitochondria were isolated also from the liver of Wistar rats starved for 24 hours.
Submitochondrial fractionation. Freshly isolated liver mitochondria were incubated with various concentrations of the detergent digitonin to solubilize mitochondrial membranes. Modified trypsin (Promega) (2 μg/1 mg protein) was used to hydrolyze the accessible mitochondrial proteins.
Separation of water soluble proteins from membrane bound proteins. 0.1 M Na_2CO_3 buffer was used to separate soluble proteins from integral membrane proteins.
Extraction of mitochondrial intermembrane space proteins. Mitochondrial intermembrane space proteins were isolated by selective disruption of the outer mitochondrial membrane by hypo-osmotic shock using 68 mosm KPi, pH 7.4. Centrifugation at 30'000 x g for 20 min at +4°C was carried out to separate the released intermembrane space proteins from the mitochondrial matrix and membrane components. The resulting supernatant was centrifugated at 200'000 x g for 30 min at +4°C to obtain a pellet which contains protein complexes with high sedimentation coefficient.
The supernatant containing mitochondrial intermembrane space proteins was briefly treated with 0.1% glutaraldehyde. Glutaraldehyde cross-links proteins in close proximity to each other. The effect of glutaraldehyde was neutralized using a high concentration of glycine (100 mM). After centrifugation at 200'000 x g for 30 min at +4°C, the resulting pellet was layered on a discontinuous CsCl gradient. Isopycnic separation was carried out by centrifugation at 200'000 x g for 16 hours at +4°C. Fractions were collected for electron microscopy studies.

β-Lactamase activity

β-Lactamase activity was measured using nitrocefin (Calbiochem) as the test substrate according to the user's protocol. Nitrocefin undergoes a change in absorbance from λ=390 nm to λ=486 nm, causing a color change from yellow to red, when the β-lactam ring is hydrolyzed. Purified GST-mLACTB (5-20 μg) and the partially purified endogenous LACTB from rat liver were used to determine their β-lactamase activity. The β-lactamase activity was monitored by measuring changes in absorbance at λ=486 nm on spectrophotometer (Parkin-Elmer). β-lactamase from *Enterobacter cloacae* (Fluka) was used as a positive control.

Fourier transform infrared spectrometry

The secondary structure of the protein was analyzed by Fourier transform infrared (FTRI) spectrometry. FTRI spectra were recorded on a Bruker Equinox 55 FTRI spectrometer equipped with a mercury-cadmium-telluride detector.

Protein concentration determination

The Bis-cinchinonic acid (BCA) assay (Pierce) was used for the colorimetric detection and quantitation of total protein. The BCA assay is a detergent-compatible method based on the alkaline reduction of the cupric ion to the cuprous ion by the protein. The Lowry method was used for low concentrations of protein since it is more sensitive. The Lowry method is based on cupric ions and Folin-Ciocalteau reagent for phenolic groups.

3.4 Proteomics (I, III)

Gel electrophoresis and immunoblotting

Proteins were separated according to their molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred onto PVDF membranes (0.2 μm, Bio-Rad). Immunoblotting was performed to determine a specific protein. Immunoreactive bands were detected by chemiluminescence (Santa Cruz Biotechnology kit) and developed using an X-ray film processor (Fuji, Inc). Antibodies used for immunoblotting are specified in the original papers **I** and **III**.

Two dimensional blue native SDS polyacrylamide gel electrophoresis

Extracted mitochondrial intermembrane space proteins were separated by two dimensional blue native SDS-PAGE electrophoresis (2D BN SDS-PAGE). In the first dimension, proteins were separated under native conditions according to their molecular weight. In the second dimension, proteins were separated according to their molecular weight by SDS-PAGE. Detailed experimental conditions of 2D BN SDS-PAGE method are described in the original paper **III**.

Matrix-assisted laser-induced ionization-desorption and time-of-flight (MALDI-TOF) mass spectrometry

Coomassie-stained bands were excised from the SDS-PAGE and 2D BN SDS-PAGE gels. Proteins were prepared by in-gel trypsinization method for analysis by MALDI-TOF mass spectrometry as described in original papers **I** and **III**. Mass spectrometry was performed

using a MALDI-TOF mass spectrometer (Bruker Autoflex III, Germany) at the Protein Chemistry Laboratory at Institute of Biomedicine, University of Helsinki.

3.5 Microscopy (III)

Electron microscopy

Postembedding immogold labeling. Biopsy samples of rat heart, kidney, skeletal muscle, spleen and liver, as well as freshly isolated mitochondria were prepared by chemical fixation in a buffer containing 0.1 M NaPi, pH7.4, 4% paraformaldehyde, 0.2% glutaraldehyde, and 0.5% DMSO. Embedding, sectioning, staining and immunolabeling were performed according to the Tokuyasu method (Tokuyasu, 1973). Cryo-sections of the tissue and isolated mitochondria were viewed using a Joel 1200 EX II electron microscope. *Whole-mount immunolabeling*. Mitochondrial intermembrane space proteins separated by CsCl-gradient centrifugation were examined by whole-mount immunolabeling. Immunolabeled whole-mount samples were negatively stained with uranyl acetate. Samples were examined under a Philips Tecnai F20 electron microscope. Detailed methods of electron microscopy (EM) sample preparation are described in the original paper **III**.

4. RESULTS

4.1 Description of LACTB

Mouse LACTB is 551 amino acids long and comprised of an N-terminal predicted mitochondrial import segment followed by a PBP and β-lactamase (PBP/β-lactamase) homology domain (fig. 10). Mouse LACTB shares around 90% amino acid identity with rat and human LACTB.

MYRLLSSVTARAAATAGPAWDGGRRGAHRRPGLPVLGLGWAGGLGLGLGLALGAKLVVGLR
GAVPIQSPADPEASGTTELSHEQALSLGSPHTPAPPAARGFSRAIESSGDLLHRIKDEVGAPGIVV
GVSVDGKEVWSEGLGYADVENRVPCKPETVMRIA***S*ISK***SLTMVALAKLWEAGKLDLDLPVQ
HYVPEFPEKEYEGEKVSVTTRLLISHLSGIRHYEKDIKKVKEEKAYKALKMVKGTPPPPDQEKE
LKEKGGKNNEKSDTPKAKAEQDSEARCRSAKPGKKKNDFEQGELYLKEKFENSIESLRLFKND
PLFFKPGSQFL***YST***FGYTLLAAIVERASGYKYLDYMQKIFHDLDMLTTVQEENEPVIYNRARFY
VYNKKKRLVNTPYVDNSYKWAGGGFLSTVGDLLKFGNAMLYGYQVGQFKNSNENLLPGYL
KPETMVMMWTPVPNTEMSWDKEGK*YAMAWGVVEKKQTYGSCRKQRHYAS***HTG***GAVGAS
SVLLVLPEELDSEAVNNKVPPRGIIVSIICNMQSVGLNSTALKIALEFDKDRAD

Fig. 10. Amino acid sequence of mLACTB. *Amino acids 1-30 – mitochondrial predicted targeting sequence; SISK, YST, HTG – catalytic site motifs. Phosphorylated serine and acetylated lysine marked with * (Kim et al., 2006; Lee et al., 2007).*

4.2 Amino acid and nucleotide sequence analysis of LACTB (II, III)

Extensive analyses were performed to characterize LACTB. The human LACTB sequence was used to search for metazoan homologs. Metazoan proteins harboring the three active site motifs (-SXXK-, -(S/Y)X(N/T)-, -(K/H)(S/T)G-) of PBP/β-lactamases were classified as LACTB family proteins (**II**, table 1). LACTB orthologs were found in all completely sequenced vertebrate genomes. In addition, LACTB orthologs were found in *Ciona intestinalis, Strongylocentrotus purpuratus, C. elegans* and *briggsae, Schistosoma japonicum*, and *Dictyostelium discoideum*.

An amino acid sequence alignment of the active site motifs and their flanking regions was performed. The analysis of amino acid sequence alignment showed that the active site motifs and several amino acids flanking the active site motifs are conserved in all analysed taxa from bacteria to humans (**II**, fig. 1A).

Compared to bacterial PBP/β-lactamases, the metazoan LACTB orthologs harbor a 50-100 amino acid long N-terminal extension and a 95 amino acid long insertion in the PBP/β-lactamase domain. Sequence analysis of metazoan LACTB orthologs with Mitoprot indicated that the N-terminal amino acid extension represented a predicted mitochondrial import sequence (**II**, fig. 1A). Further sequence analysis showed that mitochondrial import sequence directed to the matrix is followed by a sorting signal composed of hydrophobic

amino acids (**III**, fig. 2B). Analysis of the 95 amino acid long insertion in the PBP/β-lactamase domain revealed that the insert was enriched in charged and hydrophobic amino acids, having a high probability for coiled-coil formation (**III**, fig. 4 A, B).

The gene structure of the metazoan LACTB orthologs was analyzed (**II**, fig. 1B). The results showed that the structure of *Lactb* is similar in all metazoan LACTB orthologs. In all vertebrates the *Lactb* contained six exons. These results indicate that the gene structure of LACTB is well-conserved.

Next, the arrangement of the catalytic site motifs and conserved amino acids flanking the active site motifs in human and *C. elegans* LACTB were compared with a set of founding members of the each PBP/β-lactamase class (**II**, fig. 2). The results showed that LACTB was closely related to the class B of low molecular weight PBPs (LPBP-B). A sequence comparison of the PBP/β-lactamase domain of LACTB and LPBP-B proteins showed 16-27% sequence identity. A comparison of the LACTB amino acid sequence with the sequences of available crystallized LPBP-B proteins (see Literature overview section 1.2.2, fig. 8 a-c) revealed several conserved amino acids that are important for the polypeptide fold formation of the 3D structure (**II**, additional file 2).

4.3 Phylogenetic analysis of LACTB (II)

To gain insight into the evolutionary history of the LACTB family proteins, phylogenetic analysis was performed. The amino acid sequence of the PBP/β-lactamase domain of the metazoan LACTB family proteins, *Dictyostelium* LACTB homologs, and bacterial LPBP-B proteins were aligned. The inferred phylogenetic tree indicated that the LACTB family is divided into four groups which were named: LACTB orthologs, LACTB-like 1, LACTB-like 2, and esterase-like proteins (**II**, fig. 3). Bacterial LPBP-B proteins were intercalated with the different LACTB groups, suggesting that the LACTB family comprises of four alloparalogus protein lineages.

Interestingly, LACTB orthologs clustered together with LPBP-B proteins from free-living α-proteobacteria *Maricaulis maris*, *Oceanicaulis alexandrii*, and *Sphingopyxis alaskensis*. Similarly, esterase-like proteins clustered with a putative esterase from α-proteobacteria *Hyphomonas neptunium*. The LACTB-like group 1 and 2 proteins are clustered with the putative peptidases from α-proteobacteria *Bradyrhizobium japonicum* and *Mesorhizobium loti*, respectively. These results suggest that the LACTB family derive from α-proteobacteria.

4.4 Molecular cloning and expression of recombinant LACTB (I)

The cDNA of mLACTB was cloned for recombinant mLACTB protein expression in *E. coli*. Immunoblotting analysis revealed that *E. coli* expressed recombinant mLACTB protein (fig. 11).

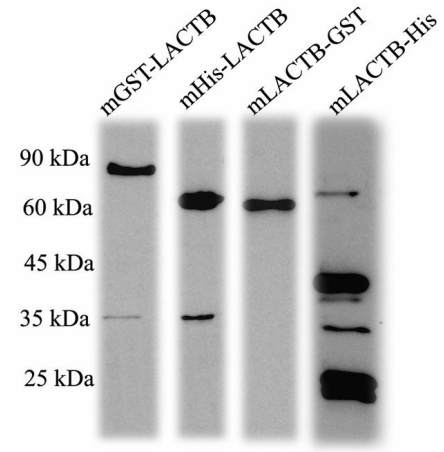

Fig. 11. Expression of recombinant mLACTB protein in E. coli. *Immunoblot analysis of mLACTB recombinant protein fused to N- or C-terminal GST or His tag. Proteins were separated on a 10% SDS-PAGE and the recombinant mLACTB protein was visualized by immunoblotting using the anti-LACTB antibody.*

One immunoreactive band corresponding to a molecular weight of 90 kDa was visualized for GST-mLACTB. However, the expression of His-mLACTB, mLACTB-GST or mLACTB-His resulted in multiple fragments or incorrect molecular weight products. MALDI-TOF mass spectrometry of the bands detected by the antibody confirmed that all products contained the C-terminus of the LACTB.

4.5 Purification of recombinant LACTB (I)

The GST-mLACTB was purified by glutathione-agarose affinity chromatography. The band corresponding to a molecular weight of 90 kDa was revealed on the SDS-PAGE gel stained by Coomassie (**I**, fig. 1). MALDI-TOF mass spectrometry verified that this band contained the full-length GST-mLACTB (**I**, fig. 2). The yield of GST-mLACTB was about 2 mg per liter of culture medium.

4.6 Secondary structure of recombinant LACTB (I)

To investigate the secondary structure of the purified GST-mLACTB, the FTIR spectrometry was performed. The FTIR spectrum showed that the GST-mLACTB was comprised of 29% α-helices, 22% β-sheets, 31% turns, and 17% random coil suggesting that the GST-mLACTB had an ordered secondary structure (**I**, fig. 3).

4.7 β-lactamase activity of LACTB

The PBP/β-lactamase homology domain of LACTB contains the serine protease SXXK motif. Therefore, the catalytic activities of GST-mLACTB and endogenous LACTB were

investigated. However, using nitrocefin as a test substrate for β-lactamase activity no enzymatic activity of the GST-mLACTB and endogenous LACTB was detected.

4.8 Subcellular localization of LACTB (III)

Proteome survey studies have given contradictive results regarding the subcellular localization of LACTB. Therefore, the subcellular localization of LACTB was tested using rat liver. Different liver fractions were analyzed by immunoblotting with anti-LACTB antibody. The immunoreactivity of LACTB was detected in the mitochondrial fraction (**III**, fig. 1C).

4.9 Submitochondrial localization of LACTB (III)

To determine the submitochondrial localization of LACTB, the biochemical and EM experiments were performed. Mitochondria were permeabilized with the detergent digitonin and subsequently treated with trypsin. Immunoblotting showed that at a digitonin concentration sufficient to permeabilize the OMM (0.3 mg digitonin per mg protein) LACTB was effectively hydrolyzed by trypsin. The same hydrolysis effect was seen for the intermembrane space proteins OPA-1 and AIF, while the mitochondrial matrix protein Hsp60 was not affected (**III**, fig. 1D).

Furthermore, immunoelectron microscopy performed on cryo-sections prepared from rat tissues and isolated mitochondria confirmed that LACTB was localized in the mitochondrial intermembrane space (**III**, fig. 1F and supplementary fig. S4). Statistical analysis of the immunogold-labeled liver cryo-sections was performed. It was found that 83% of the nanogold particles were arranged on mitochondria, especially over the cristae.

4.10 Association of LACTB to mitochondrial membranes (III)

To assess the disposition of LACTB on mitochondrial membranes, mitochondria after hypo-osmolar incubation were treated with alkali buffer ($NaCO_3$). The alkali treatment caused colocalization of LACTB together with soluble protein cytochrome c, peripherally associated with IMM, while integral membrane proteins porin and prohibitin remained in membrane pellet (**III,** fig. 1E).

4.11 Expression of LACTB in mitochondria of different rat tissues

The expression level of the LACTB protein was investigated in mitochondria isolated from different rat tissues. The highest LACTB protein expression levels were observed in the liver, testis, spleen, lacrimal and adrenal glands, and kidney mitochondria, while mitochondria from the thymus, lung and brown fat tissues had lower levels of LACTB protein (fig. 12).

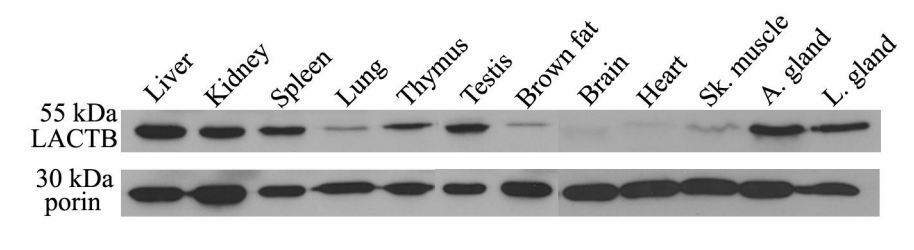

Fig. 12. Expression of LACTB protein in various rat tissues. *Mitochondrial proteins were separated on a 10% SDS-PAGE and LACTB protein was visualized by immunoblotting using the anti-LACTB and porin antibodies (unpublished data).*

Weak immunoreactive bands of higher molecular weight were detected for the LACTB protein in the skeletal muscle, heart and brain mitochondria.

4.12 Expression of LACTB during metabolic perturbations

Several studies suggest that LACTB is involved in metabolic processes. Therefore, the expression level of LACTB was tested during metabolic perturbations. First, the expression level of LACTB was investigated in the liver of rats starved for 24 hours. However, no significant difference in the LACTB protein expression level was observed between the starved rats and the control rats. Second, the expression level of LACTB protein was investigated in the liver of ethanol-treated mice. Immunoblotting showed a profound decrease in the LACTB protein expression for mouse liver treated with an ethanol diet (fig. 13).

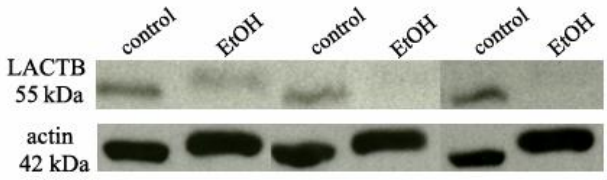

Fig. 13. Effect of ethanol feeding on the LACTB protein expression level in the mouse liver. *Mice were fed with an ethanol or a control diet for six weeks. Tissue samples from control and ethanol treated mice (n=3) were separated on a 10% SDS-PAGE and immunoblotted with anti-LACTB and actin antibodies (unpublished data).*

4.13 Expression of LACTB mRNA in various human tissues and cancer cell lines (IV)

The relative levels of LACTB mRNA expression in human tissues and cancer cell lines were determined. The highest mRNA expression levels of LACTB were found in the heart, skeletal muscle, liver and kidney, while the lowest were found in the thyroid gland, uterus and ovary (**IV**, fig. 1). Fetal tissues contained lower levels of LACTB mRNA than the corresponding adult tissues (**IV**, fig. 2). Interestingly, several cancer cell lines contained very low levels of LACTB mRNA (**IV**, supplementary table 1).

4.14 Analysis of LACTB protein under native and denaturing conditions (III)

To determine the native molecular weight of LACTB and to identify protein-protein interaction partners of LACTB, mitochondrial intermembrane space proteins were separated by 2D BN SDS-PAGE. Immunoblotting using the LACTB antibody revealed a LACTB band ranging from 600 kDa to several MDa in the native direction of the gel (**III**, fig. 2A). In the denaturing direction, this band separated into a major 55 kDa component and a minor 200 kDa component. These findings suggest that LACTB is part of a high molecular weight complex or forms a homopolymer.

MALDI-TOF mass spectrometry confirmed that both the 55 kDa and 200 kDa immunoreactive bands contained LACTB. The major 55 kDa immunoreactive band yielded a high-scoring MS/MS spectrum from peptides covering 47% of the amino acid sequence of LACTB (**III**, fig. 2B-D). Moreover, a MS/MS spectrum of a 3676.3 Da peptide was obtained. This peptide subjected to MALDI-TOF-TOF analysis could be assigned to the N-terminal 38 amino acid segment of LACTB starting from alanine 63 (**III**, fig. 2C).

In order to search for proteins that might participate in a complex formation with LACTB, the high molecular weight region of the 2D BN SDS-PAGE gel was analyzed by MALDI-TOF mass spectrometry (**III**, fig. 2A). Proteins such as glutamate dehydrogenase, carbamoylphosphate synthase, and the cytosolic ribosomal proteins 60L and 40L were identified (**III**, supplementary table 1).

4.15 Structural analysis of the LACTB polymer (III)

Since LACTB migrated as a high molecular weight protein under native conditions, the possibility that LACTB forms an organized structure was investigated. The mitochondrial intermembrane space proteins separated by CsCl-gradient centrifugation were examined by transmission EM. Filaments composed of globular subunits were observed at a gradient density of 1.25-1.28 g/cm^3 (**III**, fig. 3A). The length of the filaments varied from twenty to

several hundred nanometers. Immunoblotting and MALDI-TOF mass spectrometry confirmed that this CsCl-gradient fraction contained LACTB.

To answer the question as to whether LACTB formed filaments, whole-mount immunoelectron microscopy was performed. The results showed that LACTB antibodies coupled to nanogold particles exclusively labeled the filaments (**III**, fig. 3B). To gain insight into the subunit organization of the filament, images of 128 subunits were aligned and averaged (**III,** supplementary fig. 3). According to the tilting and averaging, the subunit volume was calculated to be 360 nm^3. It was calculated that this volume can accommodate 3-5 molecules of LACTB.

4.16 Quantification of LACTB in rat liver mitochondria (III)

To estimate the amount of LACTB in mitochondria, the high molecular weight region of the 1D BN PAGE gel was analyzed by SDS-PAGE gel electrophoresis and quantitative immunoblotting (**III**, supplementary fig. S6). Based on the intensity of Coomassie stained and immunolabeled 55 kDa bands, the amount of LACTB was estimated to be 20+/-6 pmol/mg mitochondrial protein. Comparison with other mitochondrial proteins showed that liver mitochondria contain, on average, 1500 molecules of LACTB (**III**, supplementary table S2). These results indicate that 1500 molecules of LACTB can form eighteen filaments with a length of one hundred nanometers each.

5. DISCUSSION

5.1 Evolution of LACTB

The bacterial PBP/β-lactamase proteins are essential for the synthesis of the PG layer and the maintenance of cell morphology (Cabeen and Jacob-Wagner, 2005; Macheboeuf et al., 2006; Ghosh et al., 2008). In contrast, the function of the metazoan PBP/β-lactamase family proteins remains largely unknown. Recent evidence suggests that the mammalian PBP/β-lactamase homolog LACTB (Smith et al, 2001) is involved in metabolic signaling (Rome et al., 2003; Kim et al., 2006; Lee et al., 2007; Chen et al., 2008). Therefore, by clarifying the evolutionary history of LACTB may provide clues about the function of the metazoan PBP/β-lactamase homologs.

Similarity searches for LACTB in the eukaryote genome data bases revealed orthologs in vertebrate, tunicate, echinodermata, nematode, platyhelminthes, and mycetozoa genomes. However, no homologs of LACTB were found in the yeast *S. cerevisiae* or in insect *D. melanogaster*. All LACTB orthologs contain conserved catalytic site signature motifs (Ghuysen, 1991; Massova and Mobashery, 1998). Furthermore, the identification of several conserved amino acids flanking the active site motifs suggest that LACTB and the bacterial PBP/β-lactamases share common features in the secondary and tertiary structure.

Metazoan LACTB orthologs, by contrast to bacterial PBP/β-lactamases, contain a predicted mitochondrial import sequence and a 95 amino acid long insertion in the PBP/β-lactamase domain suggesting that these segments have evolved in eukaryotes. The predicted mitochondrial import sequence of LACTB suggests that LACTB is targeted to mitochondria. The 95 amino acid long insertion forms a coiled-coil alpha-helical structure. Coiled-coils are oligomerization motifs found in a variety of proteins that function as molecular scaffolds for the cell cytoskeleton (Rose and Meier, 2004). For example, a conserved sequence of a coiled-coil structure is responsible for the polymerization of cytoplasmic intermediate filaments (Oshima, 2007). Based on this observation, we propose that the predicted coiled-coil segment might contribute to the polymerization of LACTB.

Bacterial PBP/β-lactamases family proteins are classified according to the three common catalytic site signature motifs, the specific amino acids in the active site motifs and the number of amino acid residues between the three motifs (Bush et al., 1995; Massova and Mobashery, 1998). Comparing the arrangement of the active site motifs and their amino acids in metazoan LACTB with the bacterial PBP/β-lactamase classes revealed that LACTB derives from LPBP-B proteins. LPBPs act as D-alanyl-D-alanine carboxypeptidases hydrolyzing the PG (Gosh et al., 2008). LACTB groups together with the LPBP-B proteins and contains the conserved catalytic serine residue suggesting that LACTB possess a peptidase or esterase activity. Therefore, LACTB can be classified as an active-site serine enzyme.

5.2 Homology model of LACTB

Currently three proteins from the LPBP-B subclass have been crystallized: a D-alanyl-D-alanine carboxypeptidase from *Streptomyces* R61, a D-alanyl-D-alanine aminopeptidase from *Ochrobactrum anthropii*, and a transesterase from *Burkholderia gladioli* (Kelly and Kuzin, 1995; Bompart-Gilles et al., 2000; Wagner et al., 2002). These enzymes share a similar polypeptide fold. Amino acid sequence alignments of LACTB with these LPBP-B proteins revealed a high degree of amino acid conservation in the structurally critical elements of the polypeptide fold suggesting that LACTB forms a similar polypeptide fold.

Homology modeling of LACTB using the crystal structure of the D-alanyl-D-alanine carboxypeptidase from the *Streptomyces* R61 as a template supported this hypothesis. The homology model showed that LACTB shared a similar polypeptide fold consisting of α/β region and all-helical region, with the catalytic site located between them (**III**, fig. 4 C). Additionally in the LACTB model, the predicted coiled-coil segment was located on the surface of LACTB, permitting formation of a flexible loop. In summary, holomogy modeling indicates that LACTB and bacterial LPBP-B proteins share common ancestry and features in the 3D structure organization.

5.3 Evolutionary relationship of mammalian LACTB and bacterial PBPs

Phylogenetic analysis revealed that the LACTB family is organized into four alloparalogus lineages deriving from four separate bacterial LPBP-B subclass genes. Since LACTB orthologs clustered together with LPBP-B proteins from free-living α-proteobacteria, the ancestors for the LACTB family most likely were acquired simultaneously from early α-proteobacterium. It is possible that these α-proteobacteria harbored four LPBP-B genes encoding structurally related proteins with different biochemical functions. However, during eukaryotic evolution some of these genes were eradicated from the α-proteobacterial genome following endosymbiosis (Boussau et al., 2004). This would explain why LACTB-like proteins 1, in contrast to LACTB-like proteins 2, do not appear in nematodes, while LACTB orthologs are widely distributed among the metazoan taxa. In addition, the lack of PG synthesis mechanism in metazoans due to a gene loss might explain the absence of enzymatic substrates for the LACTB family proteins.

Nevertheless, the homologs of bacterial PBP/β-lactamases are conserved in eukaryotes indicating that the LACTB family proteins play a significant role in eukaryotes. Several speculations can be invoked to explain why LACTB has been conserved in eukaryotes.

First, the phagocytotic feeding and hydrolysis of ingested PG would promote the preservation of PBP/β-lactamases genes in early eukaryotes. For example, *C. elegans* that feeds on soil bacteria retained the genes for hydrolysis of ingested PG. Moreover, the metabolism of vertebrates is linked to the metabolism of its gut microbe flora that release specific microbial metabolites. Germ-free mice colonized with an obese gut microbe showed an increase in total body fat which was associated with changes in carbohydrate and lipid metabolism (Turnbaugh et al., 2006). Furthermore, the obese microbe flora had

an increased efficiency to extract energy from the diet indicating that the gut microbe flora can contribute to obesity. According to these observations, a participation of LACTB in the metabolism of some yet unidentified substrate(s) deriving from commensal bacteria can not be ruled out.

Second, phagocytosis is a defense mechanism of the innate immune response during a pathogen infection. A microorganism infection triggers the innate immune system of a vertebrate through germline-encoded pattern-recognition receptors like the Toll-like receptors. Toll-like receptors are expressed on various immune cells, including macrophages, B cells and specific type T cells. These receptors are involved in the recognition of a variety of bacterial components (Akira et al., 2006). Indeed, the lipopolysaccharide of the gut microbiota has been identified as an inflammatory cause for insulin resistance, obesity and diabetes diseases (Cani et al., 2007). Lipopolysaschariде is the major component of the outer membrane of gram-negative bacteria, while PG is a major component of the membrane in gram-positive bacteria. Several pieces of evidence indicate that PG is a potent immunogen and can trigger innate immune response in mammals (Steiner, 2004; Hamilton et al., 2006). Innate immune response in *C. elegans* has been triggered after fungal infection (Pujol et al., 2008). In this study, a PBP homolog *Lact-1* was up regulated after fungal infection. Pujol et al. speculated that LACT-1 may be involved in pathogen recognition. Surprisingly, microarray studies have showed that *Lactb* together with other murine macrophage genes associated with cell growth and metabolism was down-regulated after *Brucella melitensis* bacterial infection (He et al., 2006). These bacteria belong to α-proteobacteria and share similar features such as the phospholipid components of the membrane and homologous genes with mitochondria (Ramirez-Romero, 1998). In addition, these bacteria suppressed expression of the mitochondrial genes involved in apoptosis activation. Based on these observations, down-regulation of LACTB might be explained by a decreased immunity response of the murine. Altogether, data suggest that LACTB has a relevant implication in metabolism and immune response of eukaryotes.

5.4 Recombinant LACTB

The expression of recombinant proteins fused to GST in *E. coli* allows production and purification of proteins. Studies have shown that recombinant GST fusion proteins expressed in *E. coli* have similar properties compared to the non-fused GST proteins, can be expressed at high levels, and are soluble (Mercado-Pimentel et al., 2002). When LACTB was expressed as an N-terminal GST fusion protein, a significant amount of the full-length GST-mLACTB was recovered by glutathione-agarose affinity chromatography. The FTIR analysis revealed that the GST-mLACTB had an ordered secondary structure. However, the GST domain of the GST-mLACTB protein itself contains 49% α-helices and 7% β-sheets (Cardoso et al., 2003), and therefore, the tag might interfere with the results of GST-mLACTB FTIR spectrum. The estimated content of α- and β-structures in the GST-mLACTB corresponds to the amount present in the bacterial class C β-lactamases (Powers et al., 2001) suggesting that the GST-mLACTB was properly folded.

When LACTB was expressed as a C-terminal GST fusion protein and as an N-terminal or C-terminal His_6 fusion protein, it underwent proteolytic degradation. Consequently, our results indicate that the N-terminal GST tag protected mLACTB from proteolysis. Furthermore, these results suggest that the N-terminal mitochondrial targeting sequence promoted proteolysis. This could be due to autoproteolysis or proteolysis by bacterial proteases. The *E. coli* strain BL21(DE3) are protease-deficient, therefore, these results support autoproteolysis. The autoproteolysis might be a maturation or an activation process. It is interesting to note, that the serine protease Omi/HtrA2 can also undergo autoproteolysis and this autoproteolytic processing of the recombinant Omi/HrtA2 protein depends on the affinity tag position (Savopoulos et al., 2000).

5.5 β-lactamase activity of LACTB

LACTB belongs to the family of PBP/β-lactamases. β-lactamases inactivate β-lactam antibiotics by hydrolyzing the peptide bond of the β-lactam ring (Ghuysen, 1991; Massova and Mobashery, 1998). However, the purified GST-mLACTB and endogenous LACTB that were tested for β-lactamase activity demonstrated no catalytic activity.

First, the absence of enzymatic activity in the GST-mLACTB and the endogenous LACTB may be due to experimental conditions. The serine protease Omi/HtrA2 shows proteolytic activity at elevated temperatures (Spiess et al., 1999). The catalytic activities of GST-mLACTB and endogenous LACTB were tested at room temperature, which possibly explains their lack of catalytic activity. Second, the absence of enzymatic activity might be due to an incorrect substrate used in the experiment. Instead of β-lactamase activity, LACTB could act as a PBP and catalyze the hydrolysis of the D-alanyl-D-alanyl peptide bond. Furthermore, the β-lactam ring of β-lactam antibiotics resembles the D-alanyl-D-alanyl peptide and binds irreversibly to PBPs. Thus, a third reason for the lack of enzymatic activity might be explained through the enzyme inactivation. Fourth, endogenous LACTB might be inactive and activated only during certain phases of cell cycle. It has been shown that *Streptoccocus pneumonia* PBP1b (Macheboeuf et al., 2005) and *E. coli* PBP3 (Eberhardt et al., 2003) are activated during cell division. Lastly, a fifth reason for the lack of endogenous LACTB enzymatic activity might be a posttranslational modification. Posttranslational modification of proteins by phosphorylation can alter the protein-protein interactions and the enzymatic activity (Lee et al., 2007). For example, the serine protease activity of Omi/HtrA2 is attenuated through the phosphorylation of Ser212 (Yang et al., 2007). Mitochondrial phosphoproteome studies revealed that the catalytic serine residue located in the -SISK- motif of LACTB is phosphorylated under normal conditions (Lee et al., 2007). Therefore, questions whether the posttranslational modification by phosphorylation may inhibit the LACTB enzymatic activity and what factors activate LACTB remain unanswered.

An esterase from *Burkholderia gladioli* (EstB) also belongs to the class of hydrolases, contains characteristic β-lactamase motifs, and displays homology to PBP/β-lactamases (Petersen et al., 2001; Weisberg et al., 2003). Site directed mutagenesis generated a variant in which the catalytic residue is located within the β-lactamase motif; however, EstB

shows no peptidase activity. Surprisingly, it is able to hydrolyze short-chain fatty acid esters and triglycerides. It is interesting to note that the polymorphism of LACTB is associated with an allergy to penicillin (Apter et al., 2008). Recent studies have shown that β-lactam antibiotics are potential therapeutic drugs to treat some neurological disorders like ischemia or amyotropic lateral sclerosis (Rothstein et al., 2005; Chu et al., 2006). However, it remains to be elucidated if LACTB plays a role in mediating the neuroprotective effect of β-lactam antibiotics. Further experimental studies are required to characterize the catalytic activity of LACTB in order to reveal if LACTB also has an enzymatic function.

5.6 Subcellular and submitochondrial localization of a soluble LACTB

The subcellular localization of the protein might give an important clue about its physiological function. Immunoblotting of a rat liver subfractions indicated that LACTB was localized in mitochondria. Investigation of submitochondrial localization of LACTB by immunoblotting revealed that LACTB like OPA1 and AIF is localized in the mitochondrial intermembrane space. Immunoelectron microscopy experiments confirmed submitochondrial localization of LACTB. Further studies done by colleagues (N. Peitsaro, A. Dapkunas, M. Linder) supported the results that LACTB is a mitochondrial protein. The immunocytochemistry experiments on HeLa cells expressing mtRFP revealed that endogenous LACTB and mtRFP shared a similar intracellular distribution (**III**, fig. 1A). In addition, a full-length LACTB and a truncated LACTB which lacks N-terminal 97 amino acids were fused to C-terminal GFP tag and expressed in HeLa mtRFP cells. The expression results demonstrated that the mitochondrial import of LACTB depends on its N-terminal amino acid sequence (**III**, fig. 1B). Immunoblotting of LACTB association with mitochondrial membranes revealed that LACTB is a soluble protein. Collectively, these results demonstrate that LACTB is a soluble mitochondrial intermembrane space protein.

In contrast to our experimental evidence, LACTB has been detected in mitochondria as part of a mitochondrial ribosomal complex (Koc et al., 2001). In this study mitochondrial ribosomes were purified from bovine liver by sucrose gradient centrifugation and all the identified proteins of unknown function were classified as ribosomal proteins. We propose that due to a similar sedimentation coefficient the LACTB migrated with the mitochondrial ribosomes.

5.7 Expression of LACTB mRNA and protein

The results of LACTB mRNA expression indicated that LACTB is expressed in all normal human tissues. The highest expression level of LACTB mRNA was observed in tissues having a high rate of oxidative metabolism suggesting that LACTB is linked to

mitochondrial function. Most cancer cell lines have an increased rate of glycolysis, an impaired oxidative phosphorylation, and are resistant to apoptosis (Gogvadze et al., 2008). The lower levels of LACTB mRNA in several cancer cell lines support the role of LACTB in energy metabolism. Altogether, the presence of LACTB in all tissues suggests that LACTB may be involved in essential cellular functions.

The results of LACTB protein expression in rat showed a different tissue distribution pattern. Conversely to high LACTB mRNA levels in the heart, skeletal muscle and brain of human tissues and rat tissues (A. Dapkunas data), the expression level of the LACTB protein was low in mitochondria of these tissues under our experimental conditions. These results suggest that LACTB might undergo different processing pathways in the heart, skeletal muscle and brain.

A first explanation for a lack of correlation between the mRNA abundance and the protein absence might involve different half-lives of proteins due to varied protein synthesis and degradation. A second reason for the different expression levels of mRNA and protein might be explained by posttranslational modifications associated with the production of protein from mRNA (Greenbaum et al., 2003). A posttranslational modification like lysine acetylation may regulate transcription, protein activity and stability. A recent study revealed that the lysine residue of LACTB is acetylated during starvation (Kim et al., 2006). It is interesting to note, that a variety of mitochondrial metabolic enzymes such as NADH dehydrogenase, isocitrate dehydrogenase, ATP synthase or glutamate dehydrogenase were also lysine-acetylated. This observation suggests that posttranslational lysine acetylation might regulate the activity of LACTB. However, no significant difference was observed in the expression level of the LACTB protein between starved and fed rats. In contrast, ethanol had a profound effect on the LACTB protein expression level in liver. The metabolic consequence of ethanol is the increased lipogenesis, an impaired fatty acid oxidation and triglyceride elimination (You and Crabb, 2004). Additional experiments are needed to investigate the relationships between ethanol, nutritional state and LACTB protein level.

It has been shown that insulin increases the LACTB mRNA level in human skeletal muscle (Rome et al., 2003). In skeletal muscle, insulin modulates the mRNA level of some important genes like hexokinase II, Glut4, phosphatidylinositol 3-kinase, or lipoprotein lipase involved in glucose and lipid metabolism pathways (Laville et al., 1996; Pentergass et al., 1998; Rome et., 2003). Impaired glucose transport and lipid metabolism due to the elevated levels of fatty acid metabolites cause insulin resistance (Lowell et al., 2005). In turn, the insulin resistance may lead to type 2 diabetes which is usually associated with obesity (Kahn et al., 2006; Muoio and Newgard, 2008). Analysis of adipose tissue transcription profile of obese co-twins showed that genes involved in the mitochondrial branched-chain amino acid catabolism, fatty acid synthesis and β-oxidation pathways were down-regulated (Pietiläinen et al., 2008). Recently, *Llp* and *Lactb* were validated as obesity genes in complex metabolic disease traits (Chen et al., 2008). *Llp* knockout and *Lactb* transgenic mice had 20% increases in fat mass compared to the lean controls. Based on the evidence that the lipoprotein lipase is responsible for the hydrolysis of triglycerides into glycerol and fatty acids (Preiss-Landl et al., 2002), LACTB possibly is involved in fatty acid metabolism. Indeed, most recent studies analyzing altered genes in *Lactb* transgenic mice showed down regulation of fatty acid metabolic pathway (Yang et al., 2009).

5.8 Proteolytic processing of LACTB

The majority of nucleus-encoded mitochondrial proteins are translated on cytosolic ribosomes as precursors with N-terminal presequences that are proteolyticaly removed by specific processing peptidases during mitochondrial import (Gakh et al., 2002). Our results show that a 55 kDa mature LACTB is formed by the removal of 62 amino acids from the N-terminus of the LACTB preprotein. This proteolytic cleavage results in the N-terminus sequence starting from the amino acids: alanine, valine, proline, and isoleucine (AVPI). A hydrophobic sorting signal located in the presequence close to the cleavage site of the LACTB preprotein suggests that LACTB is processed by two-step cleavage: first, the matrix-targeting presequence is cleaved by mitochondrial processing peptidase; second, the intermembrane space-sorting signal is removed by the inner membrane peptidase.

For example, the Omi/HtrA2 precursor protein is imported into mitochondria, the hydrophobic transmembrane domain arrests it in the IMM, and the mitochondrial processing peptidases release a soluble mature Omi into the intermembrane space (Kadomatsu et al., 2007). LACTB like Omi/HtrA2 has a prokaryotic origin, belongs to the serine protease family, and is located in the mitochondrial intermembrane space (Vandenabeele et al., 2005; Vande Walle et al., 2008). It is possible that LACTB and Omi/HtrA2 proteins share similar mitochondrial import and processing pathways.

A data base analysis of the LACTB sequence predicts that the mitochondrial processing peptidases would recognize R61 and cleave at the G62 site. It is noteworthy that this cleavage discloses an N-terminal tetrapetide motif also found in a set of pro-apoptotic IAP antagonists (Eckelman et al., 2008). Pro-apoptotic IAP antagonists can bind to the BIRs via this N-terminal tetrapeptide motif and release active caspases (Liu, 2000; Wu, 2000). Proteins that bind to BIRs in an N-terminal IAP binding motif-dependent manner include: caspases (Srinivasula et al., 2001), *Drosophila* proteins Hid, Grim and Reaper (Bangs et al., 2000), Smac/Diablo (Verhagen et al., 2000), Omi/HtrA2 (Hegde et al., 2002), glutamate dehydrogenase (GDH), Nipsnap 3 and 4, leucine-rich pentatricopeptide motif-containing protein (LRPPR**)**, and 3-hydroxyisobutyrate dehydrogenase (Verhagen et al., 2007) (fig. 14).

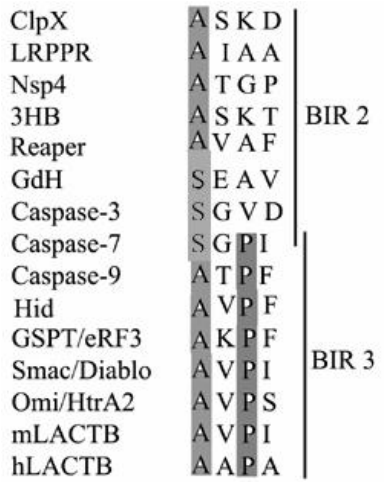

Fig. 14. Comparison of N-terminal tetrapeptide motif in mammalian and Drosophila IAP binding proteins. *Grey background represents identical amino acids. ClpX is a regulatory component of the mitochondrial Clp protease; GSPT/eRF3 is an endoplasmic reticulum associated protein. Figure modified from Verhagen et al., 2007.*

Remarkably, LACTB groups together with these pro-apoptotic IAP antagonists suggesting that LACTB could interact with the BIR domain of IAPs, although, this hypothesis needs further studies.

All known mammalian IAP binding proteins, except Smac/Diablo, appear to have other essential functions in the cell (Verhagen et al., 2007). Omi/HtrA2 acts as a chaperone (Speiss et al., 1999), LRPPR is responsible for mitochondrial RNA transcript processing (Mili and Pinol-Roma, 2003), while GDH and 3-hydroxyisobutyrate dehydrogenase are mitochondrial metabolic enzymes. Therefore, LACTB might also have multiple functions: (i) interaction with proteins involved in apoptosis through its N-terminal tetrapeptide motif and (ii) participation in metabolic signaling processes (Rome et al., 2003; Chen et al., 2008). In support of the first notion is the experimental evidence that apoptosis induces macrophage death after the phagocytosis of extracellular bacteria (Frankenberg et al., 2008). We can speculate that LACTB, in addition to its hypothetical role in the PG degradation, may act as an apoptosis-inducing signal in phagocytes.

5.9 High molecular weight complex – polymeric filaments

Analysis of mitochondrial intermembrane space proteins by 2D BN SDS-PAGE revealed that LACTB is a component of a multiprotein complex or it exists in a homopolymeric form. Most *in vivo* transient protein-protein interactions occur briefly to facilitate signaling or metabolic functions, while long-lasting protein-protein interactions favor a protein complex formation. Several proteins including GDH, carbamoylphosphate synthase, and the ribosomal subunits 60L and 40L co-migrated with LACTB. Carbamoylphosphate synthase and GDH are highly abundant proteins in liver mitochondria and like cytosolic ribosomal proteins may represent contaminants. Therefore, these findings support the hypothesis that LACTB forms a homopolymer. However, GDH has been observed to be released following tBid treatment of the liver mitochondria (Van Loo et al., 2002). This study suggests that part of the protein might be localized in the mitochondrial intermembrane space or is released under certain conditions from the matrix. Furthermore, a proteomics study of the mouse liver IMM revealed matrix proteins in their precursor form suggesting that the N-terminal targeting signals of these proteins had not yet been processed by mitochondrial matrix peptidase (Cruz et al., 2003). Interestingly, most proteins that co-migrated with LACTB participate in metabolic processes: carbamoylphosphate synthase participates in urea cycle, and GDH is a key regulatory enzyme in glutamate metabolism.

The hypothesis that LACTB forms a homopolymer was confirmed by EM studies. A CsCl-gradient fraction containing LACTB analyzed by EM revealed organized filaments. The heterogenous size of filaments suggested that a broad molecular mass range of LACTB upon electrophoretic separation might represent these filaments. Furthermore, results from the whole-mount immunoelectron microscopy confirmed that LACTB was a component of the filament. Based on analysis of the subunit organization of the LACTB filament, one subunit can accommodate 3-5 molecules of LACTB. These results suggest that the molecular weight of a subunit is 150-200 kDa. According to the molecular weight

of 200 kDa observed for the minor LACTB band upon electrophoresis, it is likely that LACTB polymer forms a homotetramer. Altogether, these results demonstrate that LACTB can polymerize and form a filament with an open symmetry. Homotrimerization of Omi/HtrA2 is mediated exclusively by the N-terminal region of the protease domain (Li et al., 2002). The oligomeric state of Omi/HtrA2 is essential for the serine protease activity suggesting that LACTB may also require polymerization for enzymatic activity.

Similar filaments were detected in the intracristal region of the intermembrane space of chemically fixed mitochondria by EM indicating that polymeric LACTB filaments exist in the intact mitochondria (**III**, fig. 3C). The results of immunoelectron microscopy of isolated mitochondria confirmed that LACTB formed clusters in the intermembrane space; however, this sample preparation method was unsuccesful to reveal the ultrastructure of the LACTB filament. Filaments observed in mitochondria were composed of globular subunits and resembled polymeric LACTB filaments. Measurements performed on these filaments and on LACTB polymers displayed a similar thickness and repeat distance (**III**). These results suggest that the isolated LACTB filaments and the intramitochondrial filaments represent the same structure. Furthermore, the observation that LACTB polymers can form *in situ* suggest that the ordered polymerization process of LACTB has a specific physiological function.

Our studies revelead that LACTB filaments localized preferentially in the intracristal region of the intermembrane space and had its ends tethered to the IMM (**III**, fig. 3C). Submitochondrial inner membrane vesicles which contain only membrane components and membrane-bound macromolecules (**III**, supplementary fig. S5) were chemically fixed and analyzed by EM. The results showed that submitochondrial inner membrane vesicles contained filaments tethered to the membrane of vesicle by its ends (**III**, fig. 3D). In conclusion, LACTB filaments form a stable module via interactions with IMM components proposing that LACTB has a structural function. In support of this notion is experimental evidence of quantitative immunobloting indicating that LACTB is abundant protein and therefore it has the potential to impact mitochondrial ultrastructure.

Consequently, the question has arisen as to if these filaments have been visualized by others. Indeed, similar helical filaments have been observed in early EM studies of mitochondria (Sasaki et al., 1991; Tandler et al., 1996). The helical filaments were localized in the mitochondrial intermembrane space of different tissues in a variety of species. The dimensional parameters of the intramitochondrial helical filaments corresponded well in the various cell types (Sasaki et al., 1991). According to discussions in previous reports, it was proposed that the helical filaments are composed of lipids or phospholipids (Svoboda and Higginson, 1964), or DNA-protein complexes (Mugaini and Walberg, 1964; Jessen, 1968). Other authors considered that helical filaments in mitochondria may emerge in consequence of a special metabolism or a metabolic disorder that result in the accumulation of abnormal proteinaceous substances that aggregate into helical filaments. Dysregulation of metabolism or metabolic disorders due to protein deficiency (Behnke, 1965), alcoholism (Iseri et al., 1966), and certain renal diseases in human (Suzuki et al., 1975) may cause mitochondria to synthesize the helical filaments (Sasaki and Suzuki, 1989; Sasaki et al., 1995). An indication that LACTB is related to metabolic disorders (Schadt et al., 2005; Chen et al., 2008; Yang et al., 2009) might support the hypothesis that metabolism dysregulation is the cause for the appearance of

filaments. Therefore, it is of interest to investigate if these helical filaments are present in obesity models.

Several groups, including ours, have seen filaments under normal conditions (Mugnaini and Walberg, 1964; Svoboda and Higginson, 1964) indicating that these filaments are not merely pathological products in the mitochondria. Filaments have been detected only in some parts of the mitochondria population within one cell suggesting that the filaments are related to a particular phase in the life of the organelle.

It should be noted, that the IMM shows a dynamic subcompartmentalization where proteins redistribute between inner boundary membrane and crista membrane depending on the physiological state of the cell (Volgel et al., 2006). The crista represents a sink for metabolites, proteins and assembly intermediates of respiratory supracomplexes (Mannella, 2006; Volgel et al., 2006). Studies revealed that ATP synthase and complex III are assembled on the crista membrane trapping the protons inside the cristae (Gilkerson et al., 2003; Strauss et al., 2008). The compartmentation of protons and ADP allows an efficient electron transfer and respiration. Furthermore, the topology of the inner membrane might influence mitochondrial functions. In addition to ATP synthase, tBid, AIF, and OPA1 oligomers can modulate the crista morphology. For example, aberrant and dilated cristae have been observed in mitochondria of AIF deficient neurons (Cheung et al., 2006). OPA1 controls apoptotic crista remodeling by segregating the cytochrome c in the intracristal space (Frezza et al., 2006). Thus compartmentation of the LACTB together with the metabolites and the proteins involved in apoptosis suggest that LACTB might play a role in core energy metabolism and apoptotic process.

Interestingly, Mootha's group assigned LACTB as a possible respiratory complex I-associated protein through phylogenetic profiling (Pagliarini et al, 2008). Our phylogenetic study supports the notion that *S. cerevisiae*, lacking respiratory chain complex I, has no homologs of LACTB. A link between LACTB and several other potential complex I-associated proteins, involved in branched-chain amino acid catabolism and lipid breakdown, further underscore the importance of LACTB in metabolic pathways.

The mitochondrial membrane architecture is highly dynamic, undergoing ultrastructural rearrangement in response to the metabolic (Hackenbrock, 1968), cell proliferation and death signals (Scorrano et al, 2002; Sun et al., 2007). It has been indicated that proteins involved in organizing the bacterial shape and division show homology to the eukaryotic cytoskeletal proteins such as actin, tubulin and intermediate filaments (review by Cabeen and Jacob-Wagner, 2005). Therefore, the question has risen if bacterial cytoskeletal proteins are linked to the PG synthesis machinery. Cabee and Jacob-Wagner suggested that PBPs might be a molecular link between the bacterial cytoskeleton and PG. Interestingly, during evolution green plants preserved some bacterial PG genes in chloroplasts. It was revealed that genes responsible for the PG synthesis pathway are essential for chloroplast division in moss (Machida et al., 2006). Both chloroplast and mitochondria evolved through endosymbiosis. Structural proteins like actin, tubulin and intermediate filaments form polymers with an open symmetry. Based on these studies, the finding that LACTB can form homopolymers suggests that LACTB possibly has a structural role in the organization of the mitochondrial supramolecular architecture. We propose that compartmentalized LACTB probably together with some other proteins forms a dynamic molecular scaffold, promoting metabolon assembly. The potential role of LACTB in the metabolon assembly might be associated with organizing enzymes for fatty acid

metabolism. We can speculate that LACTB might be involved in the regulation of the mitochondrial carnitine system, which is responsible for the fatty acid transport to mitochondria (Kerner and Hoppel, 2000). In support of this hypothesis is the observation that cystoskeletal proteins regulate carnitine palmitoyltranferase I activity (Velasco et al., 1998). In conclusion, our findings underscore the significance of the mitochondrial intermembrane space integrity for coordination processes governing both cell proliferation and death.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the main findings of this thesis, we can conclude the following:

- The full-length N-terminal GST fusion protein was obtained indicating that the expression of the *Lactb* gene in *E. coli* was successful.
- LACTB is conserved from bacteria to humans. LACTB has evolved from the penicillin binding-proteins present in the bacterial periplasmic space.
- LACTB is expressed in all normal tissues suggesting that LACTB is involved in regulating essentianl cellular functions.
- LACTB is a soluble protein localized in the mitochondrial intermembrane space. The mitochondrial import of LACTB depends on the N-terminal amino acid sequence of the protein.
- Proteolytic processing of LACTB discloses an N-terminal tetrapeptide motif shared by other mitochondrial proteins involved in apoptotic signal transduction.
- LACTB can polymerize to form filaments, possibly together with other protein(s). LACTB filaments demarcate a distinct mitochondrial microdomain. A potential role of the LACTB filaments is suggested in mitochondrial intermembrane space organization and/or mitochondrial metabolic signaling.

The importance of *Lactb*, as a new obesity-inducing gene, was revealed in a survey of complex disease traits (Schadt et al., 2005; Yang et al., 2009). Based on our findings, we propose that LACTB have multiple functions in mitochondria – (i) impact mitochondrial ultrastructure, (ii) involved in metabolism and (iii) associated with apoptosis sensing.

Structural proteins control cell shape and cell division of eukaryotes and prokaryotes (Amos et al., 2004). PBPs are required for bacteria and moss chloroplast division (Morlot et al., 2004; Machida et al., 2006). Mammalian mitochondria undergo structural changes during apoptosis (Delivani and Martin, 2006; Sun et al., 2007). These evident propose that LACTB filaments are involved in the dynamics of the mitochondrial intermembrane space. The role of LACTB in fusion-fission events remains a challenging hypothesis that needs to be further investigated.

Increasing evidences show that cellular metabolism and cell survival are regulated by growth factors that modulate the function of proteins involved in glucose metabolism and apoptosis (Hammerman et al., 2004; Heiden et al., 2001). Therefore, the main question arises whether there is a link between LACTB, mitochondrial ultrastructure, metabolism and apoptosis processes (fig. 15).

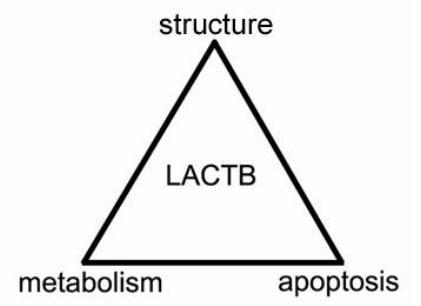

Fig. 15. Triangle – a potential role of LACTB in cell processes.

An additional hypothesis could be addressed for further understanding the function of LACTB in obesity metabolic disorder caused by inflammatory factors. The major components of the bacterial cell wall, lipopolysaccharide and PG, trigger an immune response in mammals (Hamilton et al., 2006; Cani et al., 2007) suggesting that LACTB could be implicated in innate immunity.

Further studies may answer some important questions: what benefit does the eukaryotic cell gain by retaining the LACTB, what triggers LACTB polymerization, what enzymatic substrate(s) does LACTB metabolize. Detailed knowledge of the LACTB function might offer a tool to treat mitochondria-related diseases. It is evident that LACTB is a protein that merits further study.

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