The mitochondrial ADP/ATP carrier (SLC25A4): pathological implications of its dysfunction

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LIST OF ABBREVIATIONS

ΔΨm, mitochondrial membrane potential; adPEO, autosomal dominant progressive external ophthalmoplegia; Ancp and *ANC,* mitochondrial ADP/ATP carrier and the corresponding gene; BA, bongkrekik acid; CATR, carboxyatractyloside; DCM, dilated cardiomyopahty; H or Hs, human or Homo sapiens; IR, ischemia-reperfusion; MCF, Mitochondrial Carrier Family; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; OxPhos, Oxidative Phosphorylation; Picp, mitochondrial phosphate carrier; Sc, *Saccharomyces cerevisiae*.

ABSTRACT

In aerobic eukaryotic cells, the high energy metabolite ATP is generated mainly within the mitochondria following the process of oxidative phosphorylation. The mitochondrial ATP is

exported to the cytoplasm using a specialized transport protein, the ADP/ATP carrier (SLC25A4), to provide energy to the cell. Any deficiency or dysfunction of this membrane protein leads to serious consequences on cell metabolism and can cause various diseases such as muscular dystrophy. Described as a decisive player in the programmed cell death, it was recently shown to play a role in cancer. The objective of this review is to summarize the current knowledge of the involvement of the ADP/ATP carrier in human diseases and of the efforts made at designing different model systems to study this carrier and the associated pathologies through biochemical, genetic, and structural approaches.

1. Introduction

Mitochondria provide most of the energy used by eukaryotic cells in the form of ATP, a molecule with high phosphate potential (Figure 1). Cellular energetic economy requires the continuous synthesis of ATP and an illustration is given by the amount of ATP recycled daily by each human being, which is around his or her own body mass. ATP is regenerated in mitochondria through the oxidative phosphorylation process (OxPhos). This recycling necessitates the exchange of numerous metabolites to provide the OxPhos proteins with their substrates. The bottleneck is the mitochondrial inner membrane, which is practically not permeable. Hydrophilic metabolites can cross this barrier with the aid of a family of transport proteins, the Mitochondrial Carrier Family (MCF). Among them, the mitochondrial ADP/ATP carrier (Adenine Nucleotide Carrier or Ancp) is a nuclear encoded protein, which catalyzes the exchange of ATP^{4-} generated in mitochondria by the ATP synthase with ADP^{3-} produced in the cytosol by most of the energy-consuming reactions. Therefore, Ancp is directly involved in the mitochondrial energy production in coordination with the mitochondrial phosphate carrier (Picp) since both carriers provide the ATP synthase with its substrates, ADP and Pi. The ADP/ATP exchange is not charge compensated and is driven by the mitochondrial membrane potential, $\Delta\Psi m$. The cost of this exchange amounts to around 30 % of the energy produced by mitochondrial respiration. Conversely, any dysfunction of Ancp is expected to reduce the mitochondrial energy production. The synthesis of ATP by the mitochondrial OxPhos is dependent on the coordinated expression and interaction of both nuclear and mitochondria encoded gene products. As a consequence, mutations of nuclear genes involved in mitochondrial DNA (mtDNA) maintenance are increasingly being described as associated with a variety of clinical phenotypes (Hudson et al. 2008). Indeed, a number of diseases were described for which mitochondrial disorders were associated with Ancp dysfunction and mitochondrial DNA instability. The purpose of this review is to update our understanding of Ancp at the genomic and molecular levels as well as the diseases associated with its dysfunction.

2. Genomics of the ADP/ATP carrier

Ancp is one of the most abundant mitochondrial proteins as it represents up to 10 % of the proteins of the inner membrane of bovine heart mitochondria. Ancp is encoded by four different genes; *HANC1* (or *HANT1*), *HANC2* (or *HANT3*), *HANC3* (or *HANT2*) and *HANC4* (or *HANT4*), where *H* stands for human. Their expression is tissue specific and highly regulated and adapted to particular cellular energetic demand. Indeed, *HANC* expression patterns depend on the tissue and cell types, the developmental stage and the status of cell

proliferation. Furthermore, *HANC* expression is modulated by different transcriptional elements in the promoter regions. Therefore, Ancp emerges as a logical candidate to regulate the cellular dependence on oxidative energy metabolism. *HANC1*, *HANC2* and *HANC3*, have similar genomic organization. They possess four exons and three introns at similar positions spanning a 3- to 5.9-kbp region. The corresponding cDNAs are about 1.4-kb long. The amino acid sequences of the encoded proteins are nearly 90 % identical whereas the fourth isoform is only about 70 % identical (Table 1). *HANC4* gene is spread over 44 kbp and contains 6 exons (Dolce et al. 2005). Despite their high homology, HAncp isoforms have specific kinetic and functional characteristics. The HAnc3p isoform exhibits higher molecular efficiency and V_M values than HAnc1p and HAnc2p (Table 2; De Marcos Lousa et al. 2002).

HANC1/*HANT1* is expressed at high level in heart, skeletal muscle, brain and organs with low mitotic regeneration but at a low level in proliferating cells such as myoblasts during muscle development. It is located on the sub-telomeric region of chromosome 4. Its promoter contains characteristic eukaryotic TATA and CCAAT boxes located immediately upstream of the transcription initiation site. A typical SV40 transcriptional enhancer is also found 1400 bp upstream of these boxes. The tissue-expression of the nuclear genes encoding HAnc1p and the ß subunit of the mitochondrial ATP synthase are co-regulated by the same OXBOX enhancer. Furthermore, *HANC1* expression is highly increased after completion of the proliferating phase, *i. e.* during induction of differentiation and subsequent fusion of differentiated myoblasts.

HANC2/*HANT3* is located on the pseudo-autosomal region of X and Y chromosomes. It escapes X inactivation, and is transcribed from the Y chromosome from both the active and the inactive X chromosomes (Slim et al. 1993). The *HANC2* isoform is expressed in all tissues and cultured fibroblasts at levels depending on the state of oxidative metabolism (Stepien et al. 1992). *ANC2* does not contain TATA and CCAAT boxes but only 13 potential Sp1 binding sites spread over 1250 bp. Expression of *HANC2* and *3* progressively decreases during differentiation.

HANC3/*HANT2* is located on chromosome X but unlike *HANC2*, it does not escape X inactivation. Its expression is growth-regulated and highly induced in proliferating cells, with high energetic demand, such as kidney, liver and spleen but also in cancer cells (Stepien et al. 1992). It is particularly involved in maintaining the mitochondrial $\Delta\Psi m$ and preventing apoptosis. The *HANC3* promoter shares some features with those of *HANC1* and *2*. At the onset of myoblast differentiation, *HANC3* is expressed at the same level as *HANC1* while *HANC2* expression is decreased several fold.

HANC4/*HANT4* was recently identified in both humans and mice. *ANC4* is evolutionarily conserved in mammals and it is mainly expressed in testis and in male germ cells as well as in brain and liver (Dolce et al. 2005). It is compartmentalized in the fibrous sheath in the human sperm flagellum with glycolytic enzymes (Kim and al., 2007). The targeted disruption of the *HANC4* gene in mice results in male infertility. The functional differences between *ANC4* and the other somatic *ANC* isoforms are not understood (Hamazaki et al. 2011). To date there is no evidence of *HANC4* gene mutations associated with a human disease. Eighteen *HANC4* variants have been archived in a database from large populations, including 6 non-

synonymous variants in the coding region (The 1000 Genomes Project Consortium, 2010). Further work is required to correlate these variants with pathology (Hamazaki et al. 2011). The generation of *ANC4* deficient mice resulted in the severe disruption of the seminiferous epithelium with an apparent spermatocytic arrest of the germ cell population and subsequent male infertility (Brower et al. 2009).

3. Pathophysiological aspects of the ADP/ATP carrier

Genetic defects involving the ATP synthesis can induce neurodegenerative disorders such as maternally inherited Leigh syndrome (MILS) as well as neuropathy, ataxia and retinitis pigmentosa (NARP). Oxidative phosphorylation disorders induce heterogeneous clinical aspects. Similarly, Ancp dysfunction can affect different tissues at various levels and the symptoms developed may be different from one patient to another. This was particularly true when *ANC1* was genetically inactivated in a mouse model (Graham et al. 1997). The knock-out mouse presented the characteristic features of myopathy and cardiomyopathy: cardiac hyperthrophy, mitochondrial over-proliferation in skeletal and heart muscles, ragged-red fibers and elevated levels of serum lactate. The $Ant^{-/-}$ mouse presents chronic external ophthalmoplegia but normal ocular motility. This could be the consequence of an increase in *ANC3* transcripts to compensate for *ANC1* absence (Yin et al. 2005).

HAncp defects may arise from transcriptional or translational deregulation or protein inactivation. Modification of *HANC* expression has been described in myoclonic epilepsy, which was associated with ragged-red fibers, myopathy, encephalopathy, lactic acidosis, stroke-like episodes and Kearn-Sayre syndrome. Similarly, deficiencies of the mitochondrial phosphate carrier result in severe neonatal lactic acidosis, hypertrophic cardiomyopathy and generalized muscular hypotonia (Mayr et al. 2011). This highlights the necessary intricate coordination of the oxidative phosphorylation genes resulting in a multiplicity of symptoms when one of its components is defective. Global analyses of mitochondrial transcriptomes and proteomes are now necessary along with molecular and physiological approaches to understand genetic OxPhos defects. Alterations of HAncp production were also described in the Rett syndrome (Forlani et al. 2010) and in the MFN2-related Charcot-Marie-Tooth type 2A disease (Guillet et al. 2010) underlining the complex regulation of *HANC* expression and the interlink between ATP synthesis and mitochondrial morphogenesis.

In 2002, the immunochemical analyses of skeletal and heart muscles from patients with Senger's syndrome showed a deficiency in HAnc1p protein (Jordens et al. 2002). The clinical picture observed was hypertrophic cardiomyopathy, congenital cataracts and lactic acidosis. The authors proposed that transcriptional, translational or posttranslational events are responsible for the HAnc1p deficiency associated with the Senger's syndrome.

Autoantibodies against HAnc1p were discovered in patients suffering from dilated cardiomyopahty (DCM). The cardiac defect affected both ventricles and septum, and was specific for DCM because the defect was not found in ischemic, valvular or hypertrophic cardiomyopathies (Dörner and Schultheiss, 2000). Though the HAncp content was increased, the nucleotide transport activity was decreased. The other components of the electron transport chain complexes were also not involved (Dörner et al. 2006). Rather than *HANC1*

mutation, the authors observed an isoform shift in the pattern of *HANC* expression. HAnc1p was over-produced and HAnc3p was down regulated resulting in a 50 % decrease in nucleotide transport activity. The increase in HAnc1p amount was not sufficient to compensate for a decrease in HAnc3p, which has the highest V_M value for the nucleotide exchange (De Marcos Lousa et al. 2002). Indeed, *HANC3* expression is higher in tissues predominantly expressing *HANC1* and high energy consuming, such as the heart (Dörner et al. 2006). Furthermore, *ANC3* knock out is lethal in mouse highlighting the importance of this isoform (Kokoszka et al., 2004). Altered expression of *ANC* isoforms and decreased ATP synthase activity in skeletal muscle mitochondria were also described in a dog model of heart failure (Rosca et al. 2009). Modification of Cys^{56} of HAnc1p by nitroalkenes confers acute cardioprotection during ischemia-reperfusion (IR) injury by effects on either the mitochondrial permeability transition pore (mPTP, see below), or the ADP/ATP transport or proton leak (Nadtochiy et al. 2011).

The direct involvement of a human Ancp mutation has been described so far in only one case of mitochondrial myopathy associated with cardiomyopathy (Palmieri et al. 2005). It converts the conserved Ala^{123} into an aspartic acid, introducing a negative charge in helix 3 and the mutant protein has no transport activity *in vitro*. This mutation is recessive and associated with large mitochondrial DNA deletions. HAnc1p mutations were also described in five cases of autosomal dominant progressive external ophthalmoplegia (adPEO). adPEO is a mitochondrial disorder with disease onset in early adulthood and clinically characterized by ptosis and progressive muscle weakness, most severely affecting the external eye muscles, dysphagia, dysphonia, goiter and various neurological disorders. It is associated with the mutation of six different nuclear genes, which encode the proteins Anc1p, Twinkle, POLG, POLG2, OPA1 and p53R2 (Tyynismaa et al. 2009 and references therein). Direct involvement of HAnc1p mutations is described in one sporadic (Val^{289} to Met) and in four familial (Ala^{90}Asp, Ala^{114}Pro, Leu^{98}Pro, Asp^{104}Gly) cases of adPEO (Figure 2A and B; for a review see Trézéguet et al. 2008). Three of the mutated residues (Ala^{90}Asp, Leu^{98}Pro and Ala^{114}Pro) are located at the membrane-protein interface in the putative HAnc1p structure; all of them are located near the cytosolic side of the protein (Figure 2A and B). The patients affected with adPEO carry wild type and mutant alleles. Therefore there are probably two pools of mitochondrial HAnc1p thus hampering dimerization of the non-mutated carrier (Kaukonen et al. 2000). Heterologous expression of the Ala^{114}Pro mutant *HANC1* gene in yeast caused a respiratory defect (De Marcos Lousa et al. 2002). The substitution would induce an additional bend disrupting the third transmembrane α-helix domain at the entrance of the putative pore in the putative HAnc1p structure (Figure 2B). However, the mutant HAnc1p is not produced in yeast, precluding further interpretation (De Marcos Lousa et al. 2002).

A challenging question remains unanswered: how do HAnc1p defects induce mtDNA depletion? Several hypotheses have been proposed such as Ancp involvement in maintenance of mitochondrial dNTP pool (Kaukonen et al. 2000), and its putative role in mitochondrial permeability (Chen, 2002). An increase in oxidative stress (through an increase in reactive oxygen species) as a consequence of impaired mitochondrial ATP synthesis function could result in mitochondrial membrane and DNA damages (Fontanesi et al, 2004). This needs

further investigation by deciphering genes and processes involved in mtDNA maintenance and segregation.

4. The mitochondrial Permeability Transition Pore, apoptosis and the mitochondrial ADP/ATP carrier

Ancp is a key metabolic link between mitochondrial matrix and cytosol (ADP/ATP exchange). It participates in many models of mitochondrial apoptosis by forming the mitochondrial permeability transition pore (mPTP). The massive swelling of mitochondria induced by calcium overload was first observed in mitochondria isolated from liver in the mid-1960s (Chappell and Crofts, 1965). This was due to the opening of a nonspecific channel located in the mitochondrial inner membrane (Hunter et al. 1976) and called the permeability transition pore of mitochondria (mPTP). Its opening induces a rapid loss of the intrinsic impermeability of the inner membrane, which becomes permeable to solutes up to 1500 Da. The final outcome is not only a characteristic swelling of the matrix but also the collapse of the mitochondrial membrane potential, $\Delta\Psi m$. The matrix swelling leads to the outer membrane disruption and to the leakage of molecules such as cytochrome *c*, caspases and apoptosis initiating factors (for a review see Wang and Youle, 2009). Nothing is known about the mitochondrial permeability under normal physiological conditions. During mitochondria stress, formation and opening of the mPTP forms represents for the cell a point of no return towards death, either by necrosis or by apoptotis. The pathophysiological role of the mPTP was studied in many areas, particularly in IR. Indeed, the central role of mitochondria in IR damage of the heart was hypothesized in the late 1980s (Crompton et al. 1987) and confirmed in isolated cardiomyocytes (Nazareth et al. 1991) as well as in perfused rat hearts (Griffiths and Halestrap, 1993).

Although mPTP is now well accepted, its exact nature and composition are still widely debated. The channel is thought to be formed by a super-molecular complex of proteins of the outer and inner membranes and of the intermembrane space, constituting a voltage- and Ca^{2+}-dependent pore sensitive to cyclosporine A (CsA-sensitive high-conductance channel). The anti-apoptotic role of the HAnc3p isoform was reported by Stepien and co-workers, who showed increased cell death, *in vitro* and *in vivo,* by RNA interference experiments (Le Bras et al, 2006). Ancp was one of the first proteins suggested to be an integral part of mPTP (Hunter and Haworth, 1979), along with both cyclophilin D and the mitochondrial porin, based on the observation that carboxyatractyloside (CATR) is able to induce opening of the pore. This is unlike ADP which causes an inhibition potentiated by the addition of bongkrekik acid (BA) (Hunter and Haworth, 1979; Le Quoc and Le Quoc, 1988). BA and CATR are highly specific Ancp inhibitors. This suggested that the permeability transition is related to the conformation of Ancp. Halestrap group’s suggested that the ADP/ATP carrier acts as an intermediary in the interaction between cyclophilin D and the mPTP (Halestrap and Davidson, 1990; Woodfield et al. 1998). In addition, Ancp reconstitution into liposomes in the presence of a large quantity of calcium could reversibly turn into a leaky non-selective channel (Brustovetsky and Klingenberg, 1996). However Di Lisa and Bernardi (2006) extensively discussed participation of Ancp in this phenomenon and considered that it was unlikely on the basis of genetic approaches (Kokoszka et al. 2004). From analysis of the current literature, it appears that Ancp is a regulatory element of the mPTP rather than part of its core structure

(Juhaszova et al. 2008). It is assumed that the mPTP is located at contact sites between both mitochondrial membranes but its exact molecular nature still remains to be unraveled

It was proposed that this modulation could take place through phosphorylation of HAnc1p. Indeed, during phosphosproteome analysis of isoflurane-protected mitochondria, a novel phosphorylation site was detected in HAnc1p at residue Tyr^{194} and associated with cardioprotection. The substitution of Tyr^{194} with phenylalanine, mimicking the non-phosphorylated state, could not rescue yeast growth on non-fermentable carbon sources. The author stated that phosphorylation of Tyr^{194} by the kinase Src would be essential to the ADP/ATP transport by HAnc1p (Feng et al. 2010).

5. Cancer

In a healthy cell, the main supply of energy is oxidative phosphorylation (OxPhos). Cancer cells catabolize nutrients differently since they employ aerobic glycolysis. In most cancer cells, the acquisition of a glycolytic metabolism profile would be due to hypoxic adaptation even though up to 80 % of ATP production arises from OxPhos in cancer cells (Moreno-Sanchez et al. 2007; Stubbs and Griffiths, 2010). It was hypothesized that disrupting OxPhos would interrupt the energy supply and therefore cancer cell progression. Synthetic and natural inhibitors of HAncp are currently under investigation and are reviewed in Ramsay et al. (2011).

Under normal physiological conditions, the ADP/ATP carrier exchanges cytosolic ADP^{3-} against matrix ATP^{4-} through the inner mitochondrial membrane and the transport direction is driven by the membrane potential $\Delta\Psi m$ created by OxPhos. In the condition of impaired OxPhos, a slow growth cell could be linked to the low $\Delta\Psi m$ generated by the adenine nucleotide carrier importing ATP^{4-} in the mitochondrial matrix against ADP^{3-} (Giraud and Velours, 1997). In absence of this potential, the ADP/ATP carrier can exchange either ADP or ATP for each other. *HANC3* is specifically expressed either in undifferentiated cells or in tissues that are able to proliferate and regenerate (Stepien et al. 1992; Dolce et al. 2005). *HANC3* is strongly over expressed in various types of human cancer cells (Heddi et al. 1994; Chevrollier et al. 2005; Le Bras et al. 2006). All these data lead to the assumption that *HANC3* was a potential therapeutic target against cancer (Chevrollier et al. 2010). Indeed, Stepien proposed that HAnc3p operates the reverse transport by returning the glycolytic ATP into the mitochondria (Stepien et al. 1992). The mitochondrial ATP-Mg carrier (ScaMC) exchanges $(ATP\text{-}Mg)^{2-}$ for HPO_4^{2-} or $HADP^{2-}$ between the cytosol and the mitochondrial matrix (Traba et al. 2011), and transport is absolutely dependent on the presence of extramitochondrial Ca^{2+}. However, it remains unknown whether the small divergence of *HANC* isoforms may also implicate a differentiation in the physiological function of the proteins and in their tissular distribution. It seems that the promoter region of the various isoform genes may be the key in regulating the nucleotide transport function. Indeed, a comparative study in yeast showed that the heterologous expression of human isoforms 1 to 3 under the control of *ScANC2* regulatory sequences, allowed *in vivo* production of the three HAncp and all of them restored yeast growth under respiratory conditions. This indicated that the three isoforms could efficiently perform the matrix ATP^{4-} export against the cytosolic ADP^{3-} with similar physiological characteristics (De Marcos Lousa et al. 2002).

6. Structure-functions relationships

Two natural poisons, carboxyatractyloside (CATR) and bongkrekic acid (BA) have facilitated extensive studies of Ancp. They were utilized as tools for characterizing different conformational states of the carrier. CATR is a heteroglucoside produced by the thistle *Atracylis gummifera*. It is not permeant and binds exclusively on the cytosolic side of Ancp, with a K_d value in the nanomolar range. BA is a polyunsaturated long chain fatty acid secreted by the bacteria *Pseudomonas cocovenenans*. It is permeant and binds exclusively on the matrix side of Ancp, with also a K_d value in the nanomolar range. Their binding is mutually exclusive and these properties provided the opportunity to show that Ancp adopts at least two conformations referred as CATR and BA conformations. Ancp naturally exists in the mitochondrial membrane in equilibrium between both conformations, and it is able to shift to stable complexes by inhibitor binding. In the absence of inhibitors, the interconversion can be triggered only by transportable nucleotides, suggesting these conformations are involved in ADP/ATP transport.

The elucidation of a 3D structure of the bovine Anc1p was a major step forward in the field of the membrane proteins and of the MCF of which Ancp is the model member (Pebay-Peyroula et al., 2003). It has been solved at high resolution (2.2 Å) in the presence of CATR by X-ray crystallography. It confirmed numerous previous results obtained by biochemical and genetic approaches (for reviews, see Nury et al. 2006 and Trézéguet et al. 2008). The structure is that of a monomer in complex with CATR located deep inside a cavity delineated by six transmembrane segments (Figure 2A and B). The cavity is widely open toward the intermembrane space and closed toward the matrix side by kinks in the odd numbered transmembrane helices. Three hydrophilic loops, oriented parallel to the membrane plane, tightly surround the carrier on the matrix side. However, to get deeper insights into the nucleotide transport mechanism, it will be necessary to obtain the 3D structure of the BA conformer and of the unliganded Ancp.

7. Model systems to study Ancp associated pathologies

Deciphering physiological and molecular consequences of Ancp dysfunction or mutations necessitates setting up simplified systems to overcome intricacy of *in vivo* environment. Three systems were mainly explored.

Graham et al. (1997) inactivated *ANC1* in mouse, thus affording a mouse model presenting the characteristic features of myopathy and cardiomyopathy (see above). It was used to study the implication of Ancp in mPTP (see above).

Cultured cells of adPEO patients express no phenotype and *HANC1* is normally not expressed in any cultured cells. For example, human HeLa cell lines from cervical carcinoma express only *HANC2* and *HANC3*. Furthermore, *HANC1* over expression induced apoptosis in earlier studies. However, recently, Feng et al. (2010) succeeded in expressing *HANC1* in Hela cells from the pcDNA3.HA vector when studying HAnc1p phosphorylation (see above), providing a convenient system to explore cellular consequences of *HANC1* mutations.

Yeast was widely used to study molecular, and to some extent cellular, consequences of HAnc1p mutation. *Saccharomyces cerevisiae* contains three homologous genes encoding the ADP/ATP carriers: *ScANC1*, *ScANC2* and *ScANC3*. The presence of multiple isoforms both in lower and higher eukaryotic species may be explained by the necessity to respond to different stimuli and requirements, among which is primarily oxygen (Santamaria et al. 2004). The *ScANC2* promoter is highly induced when yeast are transferred from fermentable to non-fermentable carbon sources (Betina et al. 1995) and it is the only isoform necessary for yeast growth with non-fermentable carbon sources or in the absence of oxygen. To investigate the structure-function relationships of the yeast carrier, a triple disrupted strain *Δanc1*, *Δanc2* and *Δanc3* was engineered (Drgon et al. 1991; De Marcos Lousa et al. 2002). It cannot grow with non-fermentable carbon sources. Such a strain can be transformed with various *ANC* genes, either on a plasmid or for recombination at the locus (see for example (Hamazaki et al. 2011). Their function can then be evaluated at first by growth complementation with a non-fermentable carbon source. Thus yeast Ancps could be extensively studied, taking advantage of myriads of molecular and genetic tools available for this organism (For reviews, see Nury et al. 2006 and Klingenberg, 2008). In a recent study of ScAnc2p conformation, the $Met^{254}Ala$ mutation gave rise to spontaneous intragenic second-site reversions, which restored yeast growth in respiratory condition (Clémençon et al. 2011). Interestingly, one of these mutations, Ala^{105}, involved an amino acid that is perfectly conserved among Ancp sequences and mutated into an aspartic residue in a case of adPEO (Deschauer et al. 2005).

The yeast system has the advantages of safe manipulation, rapid growth in simple and inexpensive culture conditions, and easily accessible genetics tools. Furthermore many of the cellular and metabolic processes found in higher eukaryotes are conserved in yeast species, including eukaryotic post-translational modification and secretion pathways. Such a system was explored by De Marcos Lousa et al. (2002) to characterize the kinetic properties of the human Anc1p, Anc2p and Anc3p (Table 2). However, it presents some limitations. Indeed, no mutant HAnc1p associated with adPEO is produced in *S. cerevisiae* in spite of normal amount of mRNA. The overall amino acid conservation between HAnc1p and ScAnc2p (Figure 2C) and the fact that they have similar cellular roles led several authors to study adPEO mutations in ScAnc2p (Kaukonen et al. 2000; Chen, 2002; De Marcos Lousa et al. 2002; Fontanesi et al. 2004; Palmieri et al. 2005). For example, Ala^{114} of HAnc1p corresponding to Ala^{128} of ScAnc2p was mutated into proline. Regrettably, $Ala^{128}Pro$ has variable effects depending on the authors who observed either complete inactivation of ScAnc2p or subtle modification of transport kinetic parameters. Studying the $Val^{289}Met$ mutation led to similar variability of the results. Therefore such an approach needs improvements to be routinely used and to provide relevant data interpretation. Other organisms closer to humans than yeast could be more appropriate for such studies.

However, an *S. cerevisiae* model is not completely irrelevant since it was recently validated as a model to screen drugs active against human mitochondrial disorders (Couplan et al. 2011). They identified about 10 out of 12,000 compounds from various chemical libraries, potentially active in the treatment of inherited mitochondrial diseases caused by ATP synthase deficiency. Potential therapeutic agents against mitochondrial diseases can be evaluated with growth media where respiration is required.

8. Conclusion

Mitochondria are a major site of reactive oxygen species generation, the excess of which may be damaging to the cell, and there are now numerous lines of evidence of mitochondrial dysfunction contribution to the development of metabolic disorders. Considering the fundamental role of Ancp to provide the cellular energy source, many investigations have focused on this protein to decipher its role in mitochondrial pathologies. As confirmed by this overview, Ancp implication is large and it is a promising therapeutic target.

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Table 1. *Percent of amino acid identity between the four human ADP/ATP carriers*. The alignment was realized with the Clustal W 2.1 program (Larkin et al, 2007) using the PAM matrix. The NCBI Reference Sequence numbers are NP_001142.2 (HAnc1p), NP_001627.2 (HAnc2p), NP_001143.2 (HAnc3p) and NP_112581 (HAnc4p).

%	HAnc1p (298 aa)	HAnc2p (298 aa)	HAnc3p (298 aa)	HAnc4p (315 aa)
HAnc1p	100	88	89	72
HAnc2p		100	92	70
HAnc3p			100	70
HAnc4p				100

Table 2. *Kinetic properties of the three human Anc1p to 3 expressed in S. cerevisiae and measured on isolated mitochondria*. Adapted from De Marcos Lousa et al (2002). Kinetic parameters of the wild type HAnc4p could not be measured since the wild type HAnc4p cannot be produced in yeast (Hamazaki et al 2011)

	ATR binding		Exchange of externally added ADP	
	ATR_{Max} (pmol/mg prot.)	K_d (nM)	V_M (nmol ADP/min/mg prot.)	K_M^{ADP} (µM)
HAnc1p	174	34	32.6	3.7
HAnc2p	230	22	40.4	2.5
HAnc3p	161	49	80.5	8.4

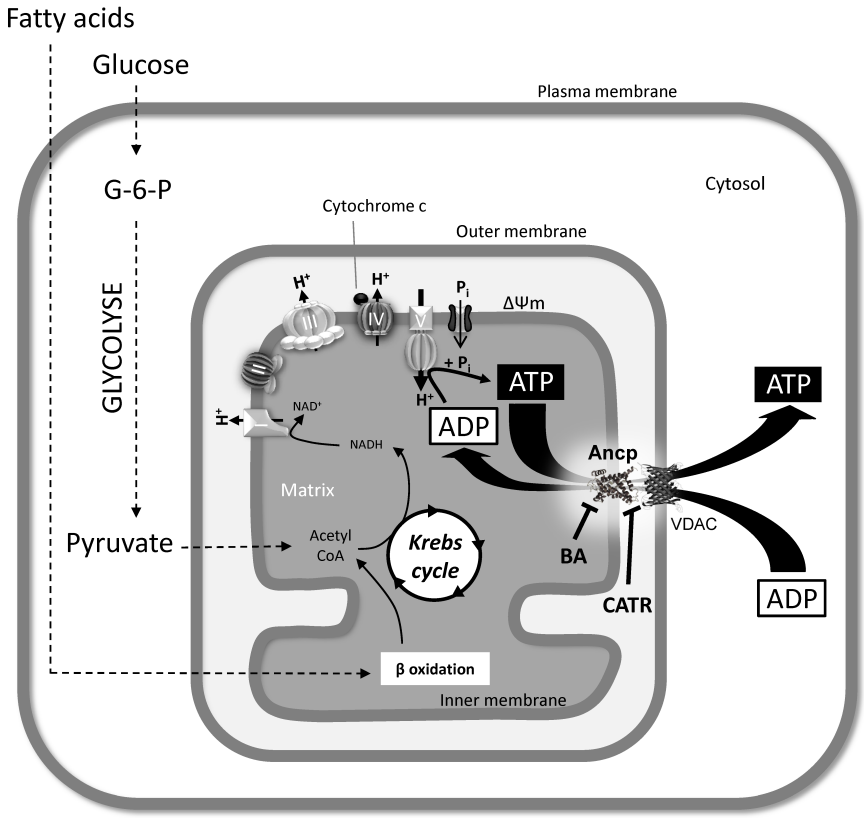

Figure 1: **Schematic representation of the metabolic key link, the ADP/ATP carrier, during ATP production in aerobic eukaryotic cells.**
In eukaryotic cells, aerobic ATP synthesis following glucose and lipid catabolism takes place mainly in mitochondria. The role of respiratory chain (formed by complexes I, II, III, IV) is to mediate proton (H^+) translocation across the mitochondrial inner membrane to establish an electrical potential and pH gradient ($\Delta\Psi m$) that drives ATP synthesis by ATP synthase (Complex V) through H^+ reuptake. The process of oxidative phosphorylation permits to generate ATP from ADP and Pi, and the ADP/ATP carrier (Ancp) plays a key metabolic role by providing an energetic link between mitochondria and the cytosol to carry out cellular functions. CATR (carboxyatractyloside) and BA (bongkrekic acid) are two very potent and specific Ancp inhibitors.

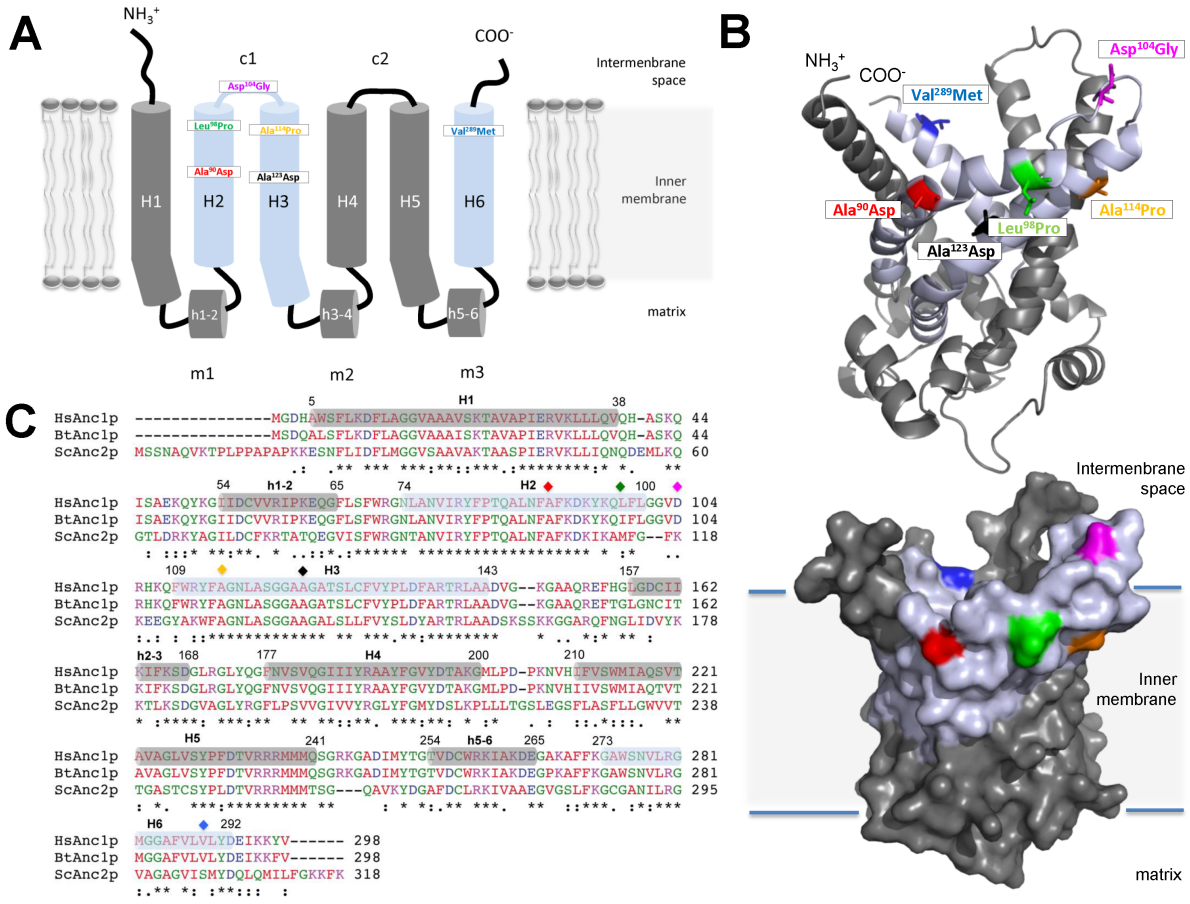

Figure 2: **3D structure model of the human ADP/ATP carrier.**

A) Schematic topology of the human Anc1p. This representation is based on a putative 3D structure generated by the PHYRE server (Kelley and Sternberg, 2009) based on the 3D structure of the bovine Anc1p (PDB ID: 1OKC) as template. The obtained topology model of the human Anc1p has six transmembrane helices (H1 to H6), three matrix loops (m1, m2, m3), each one containing a small hydrophilic helix (h1-2, h3-4 and h5-6), and two cytosolic loops (c1 and c2). The regions of pathology-associated mutations are colored in *light blue* (H2, c1, H3 and H6). The mutations involved in clinical phenotypes are indicated in different color Ala^{90}Asp (*red*) Leu^{98}Pro (*green*) Asp^{104}Gly (*purple*), Ala^{114}Pro (*orange*), Ala^{123}Asp (*black*) and Val^{298}Met (*blue*).

B) Predicted 3D structure of the human Anc1p. The predicted structure was visualized by PyMOL v0.99 software in cartoon representation (*above*). Residues involved in human diseases are represented as sticks using the same color code as in *A*. The surface representation (*below*) allows evidencing the spatial localization of these mutations in the upper third of the protein around the entrance of the cavity. The Ala^{123}Asp is localized in the cavity.

C) Amino acid sequence alignment of three Ancp. The three sequences (one-letter amino acid code) were aligned using the Clustal W 2.1 program (Larkin et al., 2007). The NCBI reference sequence numbers are the following: *Homo sapiens* isoform 1 (HsAnc1p, NP_001142.2), *Bos taurus* isoform 1 (BtAnc1p, NP_777083.1) and *Saccharomyces cerevisiae* isoform 2 (ScAnc2p, NP_009523.1). The sequences are colored according to the residue type: basic (*purple*), acid (*blue*), polar (*green*) and apolar (*red*). Identical residues are

indicated by asterisks (*) and homologous ones by two dots. Diamonds indicate positions of the mutations using the same color code as in *A*.