Muscle/heart isoform of mitochondrial adenine nucleotide translocase (ANT1) is transiently expressed during perinatal development in rat liver

Angels Grado, Carles Manchado, Roser Iglesias, Marta Giralt, Francesc Villarroya, Teresa Mampel, Octavi Viñas*

Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona. Av. Diagonal 645, 08028 Barcelona, Spain

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Abstract A postnatal increase in the content of mitochondrial ANT in rat liver which is related to the maturation of mitochondrial function has previously been reported [Schönfeld et al., Biochim. Biophys Acta 1144 (1993) 353–358]. In order to define the contribution of the ANT isoforms to this postnatal increase we have studied the expression of ANT1 and ANT2 isoforms in the liver during this period. The results show that in contrast to adult liver, perinatal liver expressed the ANT1 isoform at the mRNA and protein level, and that during this period the expression of ANT1 increased to a similar extent as total ANT content. It is concluded that the postnatal increase in ANT is mainly due to the ANT1 isoform and therefore, a role for the ANT1 isoform in the postnatal maturation of mitochondrial respiration in rat liver is suggested.

Key words: Adenine nucleotide translocator; Isoform; Perinatal liver; Respiration

1. Introduction

Adenine nucleotide translocase (ANT), or ADP/ATP carrier, is a 30 kDa nuclear-encoded mitochondrial protein, which is embedded in the inner mitochondrial membrane where it acts as a solute carrier. It is active as a homodimer or homotetramer. ANT catalyzes the exchange of ADP and ATP across the inner mitochondrial membrane, furnishing the energy-dependent reactions in the cytosol with ATP (for review see [1]). ANT has been found in multiple isoforms and these have been cloned in several species such as yeast [2–4], human [5–7], cow [8], and rat [9]. In humans there are three isoforms (ANT1–3) which show differential tissue expression [10,11]. In humans, ANT1 is predominantly expressed in heart and skeletal muscle and is considered to be a heart/muscle specific isoform, although low levels of expression have been detected in other tissues [10,11]. ANT2 is mainly expressed in tissues capable of proliferation and regeneration such as the kidneys, spleen, fibroblasts and liver [11]. Conversely, ANT3 is poorly expressed in these tissues [11]. This tissue expression pattern is similar in rodents. In both rat and mouse, ANT1 is the heart/muscle specific isoform [9,12]. It is expressed at low levels in other tissues and is almost undetectable in liver [9,12]. ANT2 is mainly expressed in liver and kidney, as in humans [9,12]. Attempts to clone a mouse or rat homologue of human ANT3 have been unsuccessful [12,13], hence either this isoform has diverged markedly among species or it is not present in rodents.

Maturation of liver mitochondrial function is achieved mainly postnatally in the rat [14,15]. The development of state 3 respiration is a bimodal process. In the first hour after birth an accumulation of adenine nucleotides in the matrix is responsible for the development of state 3 respiration [15]. However, the development of full state 3 respiration requires more time, about 1–2 days, and depends on the increase of the mitochondrial content of functional proteins. Thus, an increase of F_1F_0-ATPase within a few hours after birth has been demonstrated [15,17]. Also, an increase within the first 2 days after birth in mitochondrial ANT content correlates with the full maturation of mitochondrial function [16]. These results are reinforced by the finding that the respiratory control exerted by ANT is stronger in liver mitochondria from neonates than from adults [18]. We have studied the expression of ANT isoforms during rat liver development and we have found that in this important period for mitochondrial maturation, the ANT1 isoform is transiently expressed, suggesting that this isoform has a role in the postnatal onset of state 3 respiration.

2. Materials and methods

2.1. Cloning of rat ANT1 and ANT2 cDNA

A rat heart cDNA library (kindly provided by Nadal-Ginard, Boston, MA, USA) in lambda GT10 was screened with the full-length bovine ANT1 cDNA (kindly provided by J.E. Walker, Cambridge, UK) [8] and human ANT2 cDNA (kindly provided by R. Baserga, Philadelphia, PA, USA) [19]. 5×10^5 recombinant plaques were transferred to nylon membranes (Amersham), hybridized with radiolabelled probes at 65°C overnight and washed successively in 2×SSC and 0.5×SSC at 55°C (1×SSC is 150 mM NaCl/15 mM sodium citrate). They were autoradiographed at −70°C for 12 h with intensifying screens. After three rounds of screening, more than 40 and 15 positive individual plaques were obtained for ANT1 and ANT2 screening respectively. Ten plaques of each were selected for further investigation. Lambda DNA was purified according to standard protocols, and digested with *Eco*RI restriction enzyme and the DNA bands obtained were subcloned in pUC18 plasmid. DNA sequences were determined by the dideoxy chain termination procedure with use of the Sequenase kit (USB). With this procedure a full-length ANT1 cDNA was obtained. Due to the presence of an internal *Eco*RI restriction site in the middle of the ANT2 coding region (nucleotide 511), only a partial clone of ANT2 (about 0.7 kbp) was obtained which corresponded to the 3′ end. The sequences obtained were consistent with those reported in [9].

2.2. Animals

Female Wistar rats weighing 180–210 g were mated and the day of conception was determined by the presence of spermatozoa in vaginal smears. Fetuses were obtained by cesarian section on days 18 and 20

*Corresponding author. Fax: (34) (3) 4021559.
E-mail: ofolch@porthos.bio.ub.es

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of gestation. When the postnatal period was studied, pups remained with their mother after spontaneous delivery and were killed by decapitation at days 1 or 3 after birth. All the animals were maintained in a controlled environment (21°C, 12 h light/dark cycles). Liver and heart (when needed) were extracted and immediately processed or frozen in liquid N_2.

2.3. RNA isolation and Northern blot analysis

Total RNA was extracted using the guanidine isothiocyanate method as described in [20]. For Northern blot analysis, 20 μg of total RNA containing 0.2 μg/μl ethidium bromide was denatured at 65°C in the presence of formamide and formaldehyde, electrophoresed on formaldehyde/1.5% agarose gels and transferred to nylon membranes (Hybond N, Amersham). Transfer efficiency and the presence of equivalent amounts of ribosomal RNA in the samples were checked by ethidium bromide UV visualization. Hybridization procedures were carried out as in [21]. Filters were washed under stringent conditions (30 mM NaCl/3 mM sodium citrate, 0.1% SDS; 65°C, 30 min). Autoradiographs were quantified by densitometry.

2.4. cDNA probes

For ANT1, a fragment of 280 bp corresponding to the last 92 nucleotides of the coding region and the complete 3′ non-coding region of rat ANT1 cDNA was obtained by digestion with *Hin*dIII of a full-length clone of rat ANT1 cDNA. For ANT2, a fragment of 260 bp which corresponded to the entire 3′ non-coding region of the rat ANT2 (nucleotides 1001–1260) was obtained in the library screening. For COXII, a 0.5 kbp pIL-7 insert was obtained corresponding to part of the cDNA for subunit II of the cytochrome oxidase mitochondrial complex [22].

The cDNA probes were labeled using [α-^{32}P]deoxy-CTP (Amersham) by the random oligonucleotide priming method.

2.5. Isolation of mitochondria and Western blot analysis

Mitochondrial proteins were isolated from liver and heart according to the procedure described in [23]. The tissues washed in buffer A (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4) were homogenized in buffer B (buffer A containing 2 mM EGTA, 0.2% BSA; 10 g tissue/100 ml buffer B). The homogenates were centrifuged for 10 min at 500×*g* (4°C). The supernatant was placed on ice and the pellet was homogenized again in buffer B and centrifuged as above. Supernatants were mixed and then centrifuged at 10 000×*g* (10 min, 4°C). The resulting pellet was washed twice in buffer B and centrifuged at 5000×*g* (10 min, 4°C). Finally, the pellet was resuspended in buffer C (buffer A containing 0.5 mM EGTA, 1 mM PMSF; 1.5 ml buffer C/g tissue). Protein concentration was determined by the Bio-Rad micromethod using BSA as a standard.

For Western blot analysis, samples of mitochondrial protein (20 μg of liver mitochondrial protein and 5 μg of heart mitochondrial protein) were mixed with the appropriate amount of 5×SDS loading buffer and then heated to 95°C for 5 min and electrophoresed on SDS-15% polyacrylamide gels [24]. Coomassie blue staining of gels was performed systematically, indicating similar overall quality. Proteins were then electro-transferred to PVDF membranes (Immobilon, Millipore) and tested using two specific rabbit antisera against bovine heart ANT, kindly provided by Dr. G. Brandolin. The bound antibodies were detected with a peroxidase-conjugated affinity-purified antibody against rabbit IgG (H+L) (Bio-Rad). Immunoreactive material was detected using the enhanced chemiluminescence (ECL) detection system (Amersham). Quantification of autoradiographs was performed by densitometric analysis with Phoretix 1D.

3. Results and discussion

The sequences of rat ANT1 and ANT2 cDNA obtained

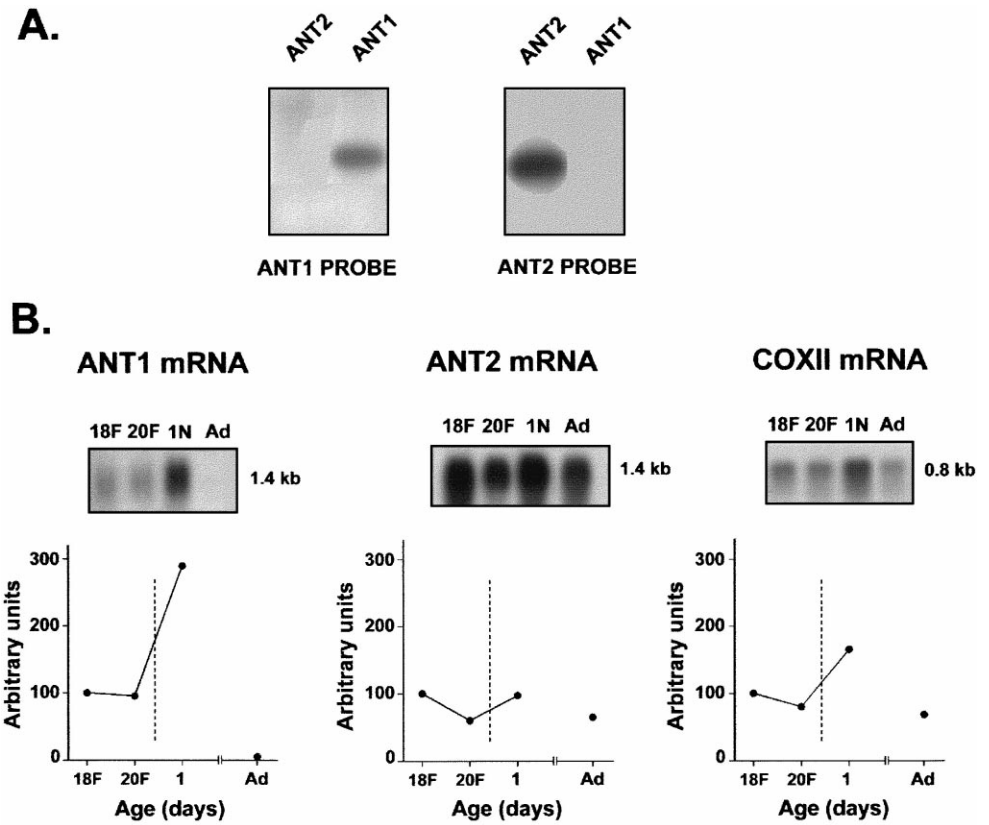

Fig. 1. A: Specificity of the cDNA probes used in Northern blots for ANT isoforms. cDNA inserts corresponding to ANT1 and ANT2 isoforms were resolved in agarose gels, transferred to nylon membranes and probed with the cDNA probes for ANT1 and ANT2. B: Representative Northern blot of total RNA from rat liver during the perinatal period probed with ANT1 and ANT2 isoforms and COXII. 18F and 20F, RNA from 18- and 20-days fetuses. 1N, RNA from 1-day neonates. Ad, RNA from adults. Quantification was performed by densitometric analysis and it is represented as the mean of at least three independent samples.

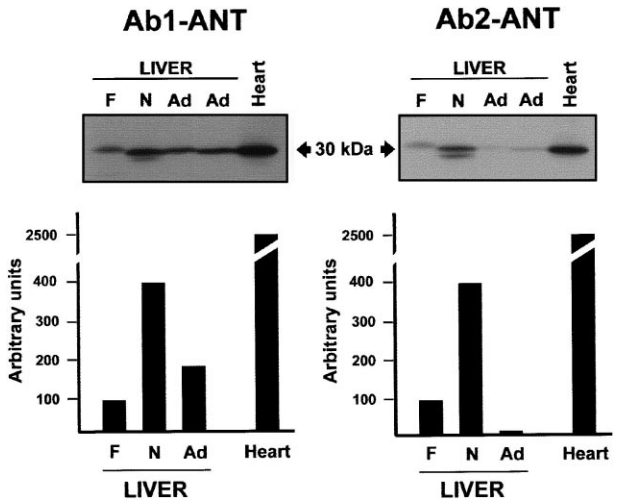

Fig. 2. Western blot of purified liver mitochondria (20 μg) from fetuses, neonates and adults and adult heart (5 μg) with two different antisera against ANT. F, 20-day fetuses. N, 3-day neonates. Ad, samples from two adults. Quantification was performed by densitometric analysis and corrected for the different amounts of mitochondrial heart protein loaded.

were the same as those reported in [9]. We chose the most divergent 3′ non-translated regions of the cDNAs as molecular probes for the measurement of the relative abundance of ANT1 and ANT2 mRNA in liver during the perinatal period. These cDNA probes were isoform specific as shown in Fig. 1A. ANT1 mRNA was expressed in fetal liver and its relative abundance increased by around three times at 1 day after birth (Fig. 1B). In adult liver ANT1 mRNA was nearly undetectable, which is consistent with previous reports [9,12]. ANT2 mRNA expression showed a 50% decrease from 18- to 20-day fetuses, recovering the value at 1 day after birth (Fig. 1B). The perinatal expression of COXII mRNA had an intermediate profile between those of ANT1 and ANT2 mRNA (Fig. 1B). These results clearly show that ANT1 mRNA is expressed in perinatal liver and that it has a marked increase over this period.

A postnatal increase in the content of ANT in liver mitochondria, which is related to the acquisition of the fully mature respiratory function that occurs in this period, has previously been reported [16]. That study was based on the specific binding of atractyloside to ANT with purified mitochondria and therefore it was not possible to identify the ANT isoform(s) responsible for this increase. We have analyzed the mitochondrial ANT content by immunodetection with two rabbit antisera raised against purified cardiac bovine ANT that show different isoform specificities. Both sera reacted strongly with the 30 kDa rat cardiac ANT (Fig. 2) as expected given the high amino acid identity ($>95\%$) between rat and bovine ANT1. Ab1-ANT was non-isoform specific and it detected a mixture of ANT isoforms. Accordingly, a specific ANT signal was obtained with liver mitochondria from fetuses, neonates and adults, with a four-fold increase from 20-day fetuses to 3-day neonates (Fig. 2). Thus, the developmental profile obtained with Ab1-ANT was consistent with the previously reported postnatal increase in the mitochondrial ANT content [16]. In contrast to Ab1-ANT, Ab2-ANT was highly specific to the cardiac ANT isoform and, accordingly, it showed little binding to ANT from adult liver mitochondria (Fig. 2). When Ab2-ANT was used with fetal and neonatal mitochondria a four-fold increase in ANT content in neonates compared to fetuses and an almost complete absence of signal in adults was observed. Comparison of the profiles obtained with both antisera showed that the neonatal increase in ANT1 isoform (Ab2-ANT) parallelled the increase in total amount of ANT (Ab1-ANT, and [16]) and therefore this increase is probably due to ANT1 with little or no involvement of ANT2. This is consistent with the changes in mRNA abundance of ANT1 and ANT2 shown in Fig. 1b. It is clear from several reports that the ANT1 isoform is predominantly expressed in terminally differentiated tissues like skeletal muscle and heart [9–11] and hence, it is considered to be a heart/muscle specific isoform. ANT2 is a growth-regulated gene which is highly expressed in a variety of proliferating cells [19,25], and consequently, it is the most abundant ANT isoform expressed in tissues capable of proliferation and regeneration such as liver or kidneys [9,11,12]. For the first time, our results suggest that the ANT1 isoform might have a function in liver specifically in the perinatal period, which is far removed from the above assumption. Some intrinsic properties of ANT1 in the transport of adenine nucleotides through the inner mitochondrial membrane or in the regulation of its expression could be important in the perinatal period when the maturation of liver mitochondrial respiration occurs. A knockout ANT1 mouse model has recently been reported [12]. These animals showed large muscle mitochondria abnormalities and cardiomyopathy according to the predominant expression of the ANT1 isoform in muscle and heart. As liver disturbances have not been studied, it would be interesting to analyze whether the maturation of mitochondrial function in the liver is affected in these animals. This would test the physiological role of the expression of ANT1 in perinatal liver.

In summary, we have demonstrated here that the muscle/heart specific ANT isoform is transiently expressed in perinatal rat liver, and that its expression parallels, both at the mRNA and at the protein level, the previously reported increase in the total amount of ANT in liver mitochondria after birth. These results strongly suggest a specific role for the ANT1 isoform in the postnatal maturation of mitochondrial respiration in rat liver.

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