

Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast *Atm1p*

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Abstract We have sequenced the entire coding region of the human ABC transporter ABC7. The protein represents a ‘half-transporter’ and displays high sequence similarity to the mouse ABC7 protein and to the mitochondrial ABC transporter *Atm1p* of *Saccharomyces cerevisiae*. As shown by immunostaining using a specific antibody, the human ABC7 protein (hABC7) is a constituent of mitochondria. The N-terminus of hABC7 contains the information for targeting and import into the organelles. When synthesised in yeast cells defective in *Atm1p* (strain $\Delta atm1/hABC7$), hABC7 protein can revert the strong growth defect observed for $\Delta atm1$ cells to near wild-type behaviour. The known phenotypical consequences of inactivation of the *ATM1* gene are almost fully amended by expression of hABC7 protein. $\Delta atm1/hABC7$ cells harbour wild-type levels of cytochromes and extra-mitochondrial heme-containing proteins, they contain normal levels of mitochondrial iron, and the cellular content of glutathione is substantially reduced relative to the high levels detected in $\Delta atm1$ cells. Our results suggest that hABC7 is a mitochondrial protein, and represents the functional orthologue of yeast *Atm1p*.

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1. Introduction

ABC transporters comprise a large family of membrane proteins that catalyse the active transfer of a variety of compounds across numerous biological membranes (for reviews see [1–4]). These proteins exhibit a conserved domain structure with two trans-membrane domains and two exposed domains harbouring an ‘ATP-binding cassette’ (ABC). In the bacterium *Escherichia coli* 57 different ABC transporters are known [5]. The majority of these proteins mediates the import of various nutritional substrates (e.g. sugars, amino acids, peptides etc.) by co-operating with specific ‘binding proteins’ in the periplasm. The remaining proteins are involved in the export of compounds such as hemolysin from the bacterial cell. In eukaryotic cells, ABC transporters have been identified in a number of cellular membranes (for review on yeast ABC transporters see [6]). They catalyse the specific transfer of hydrophobic substances, phospholipids, fatty acids, peptides and many other compounds. Only one ABC transporter has been identified so far in mitochondria, namely *Atm1p* [7,8]. Deletion of the *ATM1* gene results in a drastic growth defect of yeast cells (strain $\Delta atm1$) and a deficiency of heme-containing proteins both inside and outside of mitochondria [8].

A striking consequence of the deficiency in *Atm1p* is the drastic increase of the mitochondrial iron content [8]. This is thought to cause an oxidative stress in $\Delta atm1$ cells which is evident from the increased levels of glutathione, especially of its oxidised form, and from the hypersensitivity of *Atm1p*-deficient cells to oxidative compounds such as H_2O_2 [8]. We have suggested that the oxidative stress might cause the chemical destruction of heme attached to cytochromes and other heme-containing proteins such as catalase. While the iron-induced oxidative stress readily explains the known phenotypical appearance of $\Delta atm1$ cells, it is still unclear whether the iron accumulation in mitochondria is the primary consequence of the lack of *Atm1p*. This is mainly due to the fact that the nature of the substrate of *Atm1p* has not been identified yet.

A partial sequence (lacking the N-terminal region) of a human ABC transporter with significant homology to yeast *Atm1p* has been described recently [9]. Here, we report the sequencing of the entire open reading frame of this gene termed hABC7. The encoded hABC7 protein was identified as a constituent of human mitochondria and could be imported into isolated yeast mitochondria. When introduced into yeast cells lacking *ATM1*, the hABC7 cDNA could complement the drastic growth defect of these mutant cells. Furthermore, $\Delta atm1$ cells complemented by hABC7 displayed normal levels of cytochromes and extra-mitochondrial heme-containing proteins. Finally, the concentrations of mitochondrial iron and cellular glutathione were largely restored to wild-type levels. These data demonstrate that the hABC7 protein represents the functional orthologue of the mitochondrial ABC transporter *Atm1p* of yeast.

2. Materials and methods

2.1. Yeast strains and growth of yeast

The following *Saccharomyces cerevisiae* strains were used: strain YPH500 (*MATa*, *ura3-52*, *lys2-801*, *ade2-101*, *trp1-63*, *his3-200*, *leu2-1*) served as wild-type cells. Strain W303a (*MATa*, *ura3-1*, *ade2-1*, *trp1-1*, *his3-11,15*, *leu2-3,112*) was employed in in vitro protein import experiments. $\Delta atm1$ cells (*MATa*, *ura3-52*, *lys2-801*, *ade2-101*, *trp1-63*, *his3-200*, *leu2-1*, *atm1::LEU2*) were described earlier [8]. Cells were grown as detailed previously [8].

2.2. Production of antibody against human ABC7

A PCR fragment corresponding to the C-terminal 321 amino acid residues of hABC7 was subcloned into the *Bam*HI and *Sal*I restriction sites of pGEX-4T-2 plasmid (Pharmacia Biotech). This resulted in the fusion of the ABC domain of hABC7 to the C-terminus of glutathione-S-transferase (GST). The plasmid was transformed into *E. coli*, and used to direct the synthesis of the GST-hABC7 fusion protein after induction by 0.1 mM IPTG. After 3 h at 30°C, the cells were lysed by sonication on ice and 1% Triton X-100 was added. Cell debris was removed by centrifugation and the GST-hABC7 fusion protein was bound to a glutathione resin (Pharmacia Biotech). The

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protein was eluted with 10 mM glutathione and used for immunisation of a rabbit.

2.3. Miscellaneous procedures

Standard methods for the manipulation of DNA and for PCR were used [10]. Expression of hABC7 was achieved by transforming wild-type and Δ atm1 yeast cells with the multi-copy plasmid pRS424-GPD [11] carrying the hABC7 cDNA under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. The following published methods were used: transformation of yeast cells [12]; isolation of plasmids from yeast [10]; isolation of yeast mitochondria [13]; in vitro transcription and translation and protein import into isolated mitochondria [14–17]; isolation of mitochondria and post-mitochondrial supernatant from human liver [18]; for preparation of cytosol, the post-mitochondrial supernatant was centrifuged for 1 h at $100\,000\times g$; whole cell lysates by breaking the cells with glass beads [19]; measurement of the enzyme activity of catalase [20]; determination of total and oxidised glutathione [21]; detection of 'non-heme, non-iron-sulfur' iron after solubilisation of the mitochondria in 10 mM MOPS-KOH, pH 6.5, 1% Triton X-100 [22].

3. Results

3.1. The full-length sequence of human ABC7

The full-length sequence of human ABC7 (hABC7) was derived from an expressed sequence tag clone (EST no. AA305099) which displayed significant sequence identity to mouse ABC7 encoding an ABC transporter [9]. The entire EST clone was sequenced and the protein sequence of hABC7 protein (752 amino acid residues) was derived. During the course of our studies the full-length sequence of hABC7 has been reported by another group [23], GenBank accession no. AB005289. However, no further information on this protein was provided. The human ABC7 protein shares highest sequence homology with the mouse ABC7 partial sequence (87% identical and 91% chemically similar amino acid residues), with *S. cerevisiae* Atm1p (43% and 72%, respectively) and with an open reading frame of *S. pombe* (38% and 66%, respectively). The sequence identity between these 4 proteins is similar in the C-terminal ABC domain and in the N-terminal membrane-spanning region (about 42% for the human and *S. cerevisiae* proteins). Usually, high homology between members of the ABC transporter family is confined to the ABC domains. The significant homology also in the membrane-spanning region raises the possibility that the mammalian and the fungal proteins comprise functional orthologues.

3.2. hABC7 is a mitochondrial protein

The sub-cellular localisation of hABC7 protein was determined. We tested the ability of the N-terminus of hABC7 protein to serve as a mitochondrial targeting sequence and direct the protein to the organelles in vitro (for reviews see [24,25]). A fusion protein comprised of the first 135 amino acid residues of hABC7 and mouse dihydrofolate reductase (DHFR) was synthesised in reticulocyte lysate. The radiolabelled protein (termed preABC7(135)-DHFR) was incubated with isolated yeast mitochondria to allow import of the pre-protein. The reaction mixture was treated with proteinase K to digest the precursor protein which had not been sequestered by the organelles. A substantial fraction of the added preprotein became processed to a shorter form upon incubation with mitochondria (Fig. 1A, upper panel). The majority of this 'mature' protein was resistant to digestion by proteinase K indicating that it was imported into mitochondria and cleaved by the matrix processing peptidase. When the outer

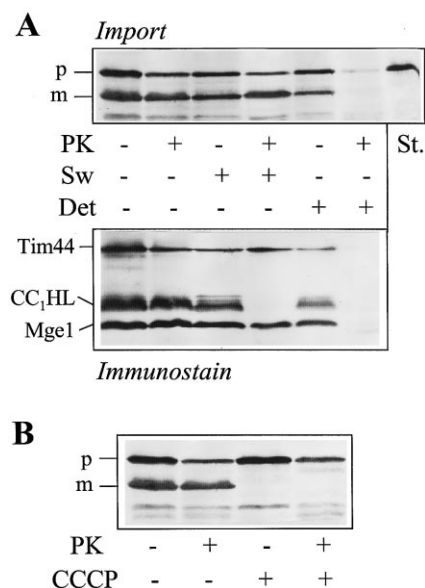


Fig. 1. The N-terminus of human ABC7 contains information for targeting to mitochondria. A: The fusion protein preABC7(135)-DHFR was synthesised in reticulocyte lysate and incubated with isolated yeast mitochondria [17]. After 15 min at 25°C mitochondria were reisolated by centrifugation. Samples were resuspended in SoH buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.2), subjected to osmotic swelling (Sw), a procedure to selectively rupture the outer membrane [41] or dissolved in 0.5% Triton X-100 detergent (Det). Samples were treated with or without 50 µg/ml proteinase K (PK) on ice for 10 min. Proteins were precipitated with TCA, separated by SDS-PAGE and blotted onto nitrocellulose membrane. Radiolabelled proteins were visualised by autoradiography of the blot (Import) which then was immunostained for the indicated marker proteins of the matrix (Tim44 and Mge1) and the intermembrane space (CC₁HL). CC₁HL was degraded by protease after rupture of the outer membrane, whereas Tim44 and Mge1 were accessible to proteolysis only after opening of both the outer and inner membranes. p and m: Precursor and mature forms of preABC7(135)-DHFR, respectively; St.: a standard containing 20% of input preprotein. B: Import of preABC7(135)-DHFR was performed as in A in the presence or absence of 50 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to deplete the membrane potential. Samples were treated with proteinase K and import was analysed as described in A.

membrane of mitochondria was selectively opened by a swelling procedure (see Fig. 1A, lower panel), the imported fusion protein was resistant to proteinase K. In contrast, upon lysis of the mitochondria with detergent the fusion protein became sensitive to proteolytic attack demonstrating that it had become imported into the matrix.

The transfer of the presequence across the mitochondrial inner membrane requires a membrane potential, $\Delta\Psi$ [26,27]. We therefore tested the dependence of the import of the fusion protein upon the existence of an energised inner membrane. The membrane potential was depleted by the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Under these conditions neither cleavage of pre-ABC7(135)-DHFR to the mature form nor protease resistance of the fusion protein was observed (Fig. 1B). A small fraction of added preABC7(135)-DHFR remained inaccessible to proteolytic degradation in these experiments which is due to aggregation of the protein (Fig. 1; not shown). Together, these data demonstrate that the N-terminus of hABC7 protein can target an attached passenger protein to mitochondria and di-

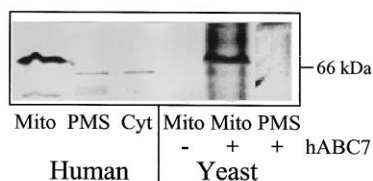


Fig. 2. Human ABC7 is a mitochondrial protein. A human liver was homogenised and mitochondria (Mito), a post-mitochondrial supernatant (PMS) and a cytosolic fraction (Cyt) were prepared (left part). Mitochondria and post-mitochondrial supernatant were isolated from wild-type yeast cells expressing or not hABC7 protein (right part). Aliquots were subjected to SDS-PAGE and immunostaining was performed using the antiserum raised against the ABC domain of hABC7. The hABC7 protein tends to aggregate after expression in yeast (see staining above hABC7 band).

rect its import into the organelles in a membrane potential-dependent fashion. This suggests that hABC7 protein represents a constituent of mitochondria.

To test the sub-cellular localisation of hABC7 protein in vivo, we raised an antibody against a fusion protein of hABC7 and glutathione *S*-transferase. The fusion protein was expressed in *E. coli* and purified by affinity chromatography using a glutathione resin. The antibody could specifically immunoprecipitate hABC7 that was synthesised in vitro (not shown). It recognised a protein of 68 kDa in mitochondria isolated from human liver, but not in a post-mitochondrial supernatant or a cytosolic fraction (Fig. 2). When hABC7 protein was expressed in wild-type yeast cells by transforming with a multi-copy plasmid encoding hABC7, a protein of the same electrophoretic mobility (68 kDa) was recognised by this antiserum (Fig. 2). No immunostaining was observed for the post-mitochondrial supernatant derived from yeast cells expressing hABC7 protein or for wild-type yeast mitochondria. In summary, our in vitro and in vivo data demonstrate that hABC7 protein is localised in mitochondria.

3.3. Human ABC7 can functionally complement the deletion of yeast *ATM1*

The hABC7 gene was expressed in yeast cells in which the *ATM1* gene had been deleted (strain $\Delta atm1$; [8]). Synthesis of hABC7 restored almost wild-type growth similar to what was found when the *ATM1* gene was reintroduced into these cells (Fig. 3A). Thus, hABC7 protein can largely compensate for the growth defects observed upon inactivation of *Atm1p*.

We further asked whether expression of hABC7 in $\Delta atm1$ yeast cells (strain $\Delta atm1/hABC7$) can revert the phenotypical consequences observed for $\Delta atm1$ cells, namely the high content of iron within mitochondria, the lack of cytochromes and extra-mitochondrial heme-containing proteins as well as the oxidative stress indicated by, for instance, increased levels of cellular glutathione [8]. Mitochondria were purified from $\Delta atm1/hABC7$ cells. The isolated organelles contained wild-type levels of cytochromes thus differing markedly from $\Delta atm1$ mitochondria which were largely deficient in all cytochromes (Fig. 3B; [8]). A similar result was obtained for the extra-mitochondrial heme-containing protein catalase. The strongly decreased level of this enzyme in $\Delta atm1$ cells was restored to almost wild-type activity upon expression of hABC7 (Fig. 3C). Further, $\Delta atm1/hABC7$ cells contained only twofold increased levels of mitochondrial 'non-heme,

non-iron/sulfur' iron as compared to wild-type organelles, whereas a large increase in iron was observed for $\Delta atm1$ mitochondria (Fig. 3D; [8]).

The cellular level of total and oxidised glutathione was determined using cell lysates. Expression of hABC7 in $\Delta atm1$ cells resulted in a substantial, but not complete reduction of the elevated levels of glutathione found in $\Delta atm1$ cells (Fig. 3E). The reason for this partial complementation is unknown, but may be related to the slightly slower growth of $\Delta atm1/hABC7$ relative to wild-type cells (see Fig. 3A). In summary, expression of hABC7 can largely or completely revert the known phenotypical consequences of the deletion of yeast *ATM1*. Our results demonstrate that hABC7 protein represents the functional orthologue of the mitochondrial ABC transporter *Atm1p*.

4. Discussion

In the present contribution, we determined the full-length sequence of the human ABC transporter hABC7. Further, we describe the localisation of hABC7 within mitochondria and the functional complementation by hABC7 of a yeast mutant lacking the mitochondrial ABC transporter *Atm1p*. The sequence similarity among various members of the ABC transporter family is limited. Highest sequence conservation is observed in the ABC domain, in particular in the region of nucleotide binding (the P-loop), whereas in the membrane spanning region similarity usually is low. The significant homology of the mammalian ABC7 proteins with the fungal *Atm1p* in both domains (see above) rendered it likely that these proteins represent functional orthologues.

We provide evidence for the localisation of hABC7 protein within mitochondria. An antibody raised against hABC7 recognised a protein of predicted size in human mitochondria and in mitochondria isolated from yeast cells expressing this protein. As demonstrated by our in vitro import studies the targeting information appears to be localised at the N-terminus of hABC7, as this is the case for most mitochondrial preproteins. It should be mentioned that the extreme N-terminal region of hABC7 differs from typical mitochondrial presequences [28,29] in that it contains an unusual number of hydrophobic amino acids and a negatively charged residue. Thus, based on sequence information it was impossible to predict a mitochondrial localisation of this protein. As estimated from the size difference of the precursor and mature forms of the fusion protein preABC7(135)-DHFR used in our in vitro import studies (about 4–4.5 kDa; see Fig. 1), the processing site may be localised after residue 43 (see [30,31]). Thus, these first 43 amino acid residues appear to represent the targeting sequence assuring the mitochondrial localisation of hABC7 protein.

On the basis of the results reported in this communication, hABC7 protein can be regarded as a functional orthologue of yeast *Atm1p* [7,8]. When expressed in yeast cells lacking *Atm1p*, the hABC7 protein was localised to mitochondria and could replace the functions of *Atm1p* to a great extent. First, it restored almost wild-type growth to $\Delta atm1$ cells. Second, the $\Delta atm1/hABC7$ cells contained wild-type levels of cytochromes and extra-mitochondrial heme-containing proteins such as catalase. Third, mitochondria did not contain the largely elevated iron levels that were reported for $\Delta atm1$ organelles [8]. Finally, the cellular content of glutathione was

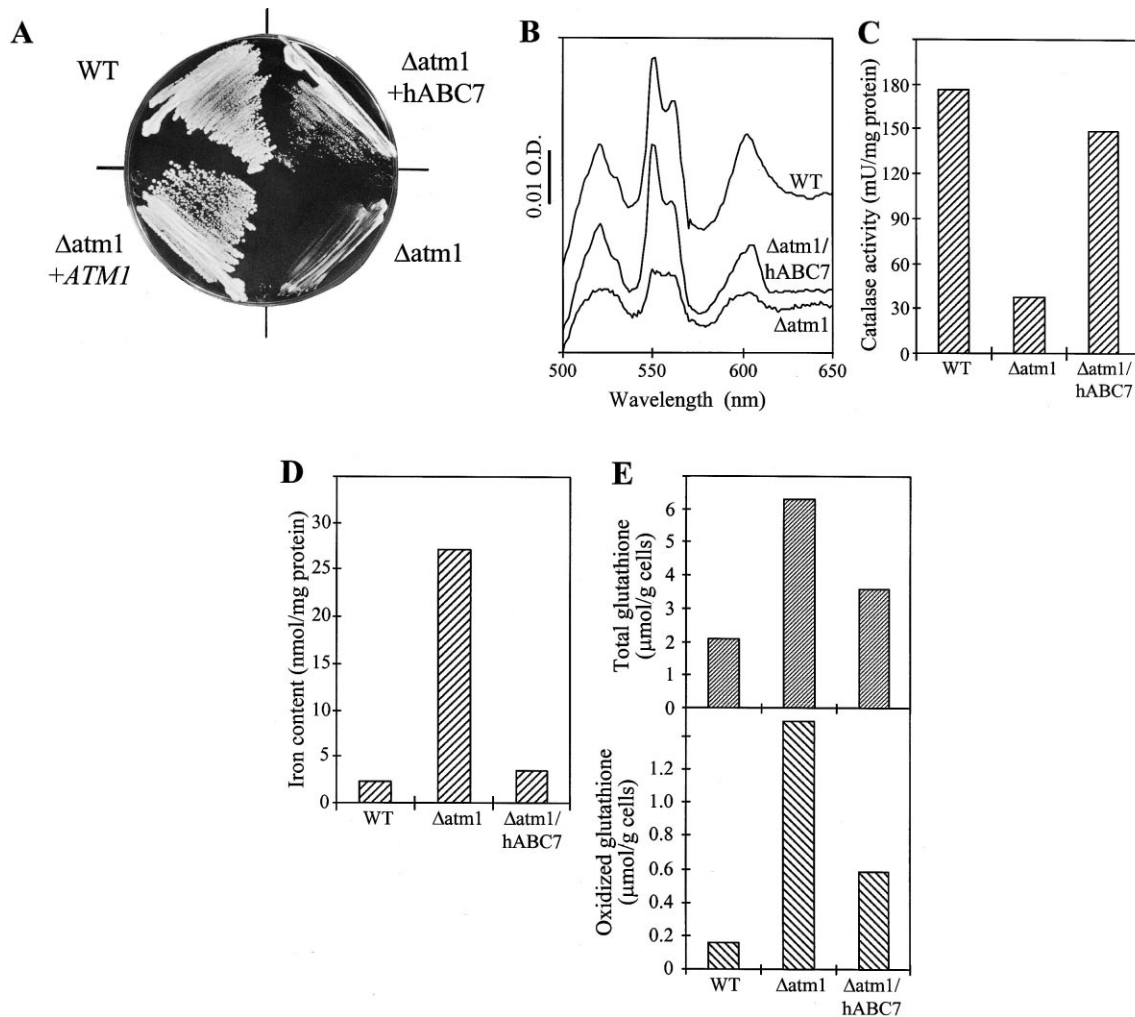


Fig. 3. Human ABC7 can largely restore the phenotypic consequences of the deletion of the yeast *ATM1* gene. A: Expression of hABC7 restores growth to $\Delta atm1$ cells. Wild-type (WT) and $\Delta atm1$ cells harbouring either an empty vector ($\Delta atm1$), the yeast *ATM1* gene or the human ABC7 gene (hABC7) on the multi-copy plasmid pRS424-GPD were grown on agar plates containing YPD medium for 3 days at 30°C. B: Cytochrome spectra were recorded using mitochondria isolated from wild-type, $\Delta atm1$ or $\Delta atm1/hABC7$ strains [8]. The bar on the left represents an absorption difference of 0.01. O.D.: optical density. C: Catalase activities were measured using extracts of the indicated cells grown in YPD medium [20]. D: The iron content of mitochondria isolated from the indicated strains grown on YPGal medium was measured as previously published [8,22]. E: The cellular contents of total and oxidised glutathione were determined for the indicated yeast strains grown on YPD medium as described [21].

substantially reduced by expression of hABC7 protein in $\Delta atm1$ cells. These data suggest that the functions of yeast Atm1p and human ABC7 overlap and that they likely transport the same substrate across the mitochondrial inner membrane.

The next challenge will be to understand the function of Atm1p and hABC7. Based on the orientation in the membrane with the ABC domain facing the matrix space [7], Atm1p/hABC7 functions as an exporter. Which substrate might require active export from mitochondria mediated by Atm1p/hABC7? Clues to answer this important question might come from the functional reconstitution of the ABC transporters and from the analysis of components interacting with these proteins. One protein which has been reported to genetically interact with Atm1p is the mitochondrial branched-chain amino acid transaminase Bat1p of yeast, which upon over-expression can restore growth to the temperature-sensitive strain *atm1-1* at the non-permissive condition

[32]. Branched-chain α -keto acids have been described to function as siderophores in certain bacteria [33]. It thus seems possible that α -keto acids may have a similar function in eukaryotic cells and alleviate the toxic effects of increased iron levels in cells defective in Atm1p. More investigations are needed to solve these interesting questions.

Mutations in human ABC transporters are the cause of a variety of diseases (see, for instance, [34]). The human ABC7 gene has been mapped to chromosome Xq13.1-q13.3 [9]. This region has been implicated in hereditary X-linked sideroblastic anemia, in which cells contain high deposits of non-heme iron within mitochondria which are arranged in ring-like structures around the nucleus (ring sideroblasts; [35]). The high iron content of yeast mitochondria deficient in Atm1p makes it likely that hABC7 might be connected to this iron storage disease. A similar increase in mitochondrial iron levels has been found in mutants of frataxin, a protein of the mitochondrial matrix [36,37]. Mutations in the human gene of

frataxin are responsible for the neuro-degenerative disorder Friedreich's ataxia [38–40]. It will be interesting to investigate a possible interaction between Atm1p/hABC7 and frataxin and to understand the role of these proteins in mitochondrial iron homeostasis.

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