M-ABC2, a new human mitochondrial ATP-binding cassette membrane protein

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Abstract We have isolated a human cDNA encoding a novel ATP-binding cassette (ABC) protein whose gene was previously localized to chromosome 1q42 [Allikmets et al. (1995) Mamm. Genome 6, 111–117]. The gene transcript is expressed in all human tissues examined, with the highest levels in bone marrow. A non-expressed pseudogene also exists at chromosome 15q13–14. The new protein, which is most similar to the mitochondrial (M)-ABC1 protein, was also localized to mitochondria and therefore designated 'M-ABC2'. The N-terminus of M-ABC2 was shown to contain a mitochondrial-targeting signal sequence.

Key words: ATP-binding cassette protein; Mitochondrion; Pseudogene; Signal sequence

1. Introduction

Members of the ATP-binding cassette (ABC) transporter superfamily, characterized by the presence of transmembrane domains (TMDs) and the highly conserved nucleotide-binding domains (NBDs), are involved in the energy-dependent transport of a wide variety of molecules across cellular membranes. Eukaryotic ABC proteins are categorized into full proteins, which contain two TMDs and two NBDs, and half proteins, which contain one of each domain [1]. Extensively studied mammalian full ABC proteins include P-glycoprotein, whose expression contributes to multidrug resistance [1], and the chloride channel CFTR, mutations of which give rise to cystic fibrosis [2]. The half ABC proteins, TAP1 and TAP2 (transporters associated with antigen processing), form a heteromeric complex that functions in the translocation of antigenic peptides from the cytosol to the lumen of the endoplasmic reticulum [3].

The growth of expressed sequence tagged (EST) cDNA databases [4] has accelerated the identification of new members of the human ABC family, for which 30 unique ABC proteins have been identified to date [5]. We recently identified the human mitochondrial half ABC protein, M-ABC1 by searching the EST cDNA database [6]. The human mitochondrial half ABC protein ABC7 has been shown to complement the growth defect in ATM1-deficient *Saccharomyces cerevisiae* [7]. Yeast cells deficient for the mitochondrial half ABC protein ATM1 accumulate high levels of iron in mitochondria, suggesting that ATM1 and ABC7 are functional orthologs involved in mitochondrial iron homeostasis. Furthermore, mutations in the human ABC7 gene on chromosome Xq13 is associated with X-linked sideroblastic anemia and ataxia, a disease characterized by elevated free erythrocyte protoporphyrin levels and lack of excessive parenchymal iron deposition [8].

We present here a third member of the human mitochondrial half protein subfamily, designated M-ABC2 (also named ABCB10, see http://www.gene.ucl.ac.uk/users/hester/abc.html). The M-ABC2 cDNA corresponds to an EST clone previously reported by Allikmets et al. [9]. A study of the chromosomal localization, mRNA tissue distribution and protein localization of M-ABC2 is presented.

2. Materials and methods

2.1. Isolation of cDNA and genomic DNA clones

The cDNA insert of EST clone 193990 (Genbank Accession No. R83875) was used to hybridization screen a human acute lymphoblastic leukemia CCRF-CEM cDNA library [6] and a human genomic DNA library (gift from Dr. Dixie Mager, Terry Fox Labs, BC Cancer Research Centre, Vancouver, BC, Canada). The nucleotide sequence for the M-ABC2 cDNA has been deposited in the GenBank database (Accession No. AF216833). The partial nucleotide sequences of the genomic DNA clones HGL9C1 and HGL9C3 (corresponding to the M-ABC2 cDNA at positions 638–1090, 1406–1697 and 2441–3180 bp) have been deposited under GenBank Accession No. AF218417, AF218418 and AF218419, respectively. DNA was sequenced manually (US Biochemicals) or on a Model 310 DNA Sequencer (Applied Biosystems). The sequence was analyzed using the Wisconsin (GCG) Package (Genetics Computer Group, WI, USA) and the TopPredII program [10].

Phylogenetic analysis was performed using ClustalX [11]. A neighbor-joining bootstrap tree was generated from the alignment using PAUP*4.0 [12]. The GenBank accession numbers for the ABC proteins employed were: TAP1, X57522; MDR1, 4505769; M-ABC1, AF047690; TAPL, AB027520; TAP2, M74447; ABC7, AB005289; UMAT, AJ003004; MDL1, U17246; MDL2, L16959; ATM1, Z49212; HMT1, AL031546.

2.2. Chromosomal mapping

Fluorescent in situ hybridization (FISH) analysis of the M-ABC2 genomic clone HGL9C1 was performed by the Human Genome FISH Mapping Resource Centre (Hospital for Sick Children, Toronto, Canada). Paired primers (5′-GAGCGTGGTGCCTCCAG-3′, 5′-ATGG-

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Abbreviations: ABC, ATP-binding cassette; EST, expressed sequence tag; FISH, fluorescent in situ hybridization; GFP, green fluorescent protein; GST, glutathione *S*-transferase; NBD, nucleotide-binding domain; RT-PCR, reverse transcription-polymerase chain reaction; TAP, transporter associated with antigen processing; TMD, transmembrane domain

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TCCACTTTGCTGGC-3′) specific to the human M-ABC2 cDNA was used for polymerase chain reaction (PCR) amplification of a monochromosome somatic cell hybrid panel (gift from Dr. Steve Scherer, Hospital for Sick Children, Toronto, Canada).

2.3. Northern blot analysis and reverse transcription (RT)-PCR amplification

The cDNA insert of EST clone 193990 was used for the hybridization of human multiple tissue Northern blots (Clontech). Total RNA was isolated using Trizol reagent (Gibco BRL), DNaseI-treated and employed in oligo dT-primed first strand cDNA synthesis. Replicate aliquots were then subjected to PCR amplification using oligonucleotides corresponding to regions in the 3′ untranslated region; one primer being common to the M-ABC2 genes on chromosomes 1 and 15 (5′-CTGTTGAATAGCAAGGAC-3′) and the second primer being specific to the chromosome 1 (5′-TTTGAACAGGAAAACATG-3′) or chromosome 15 genes (5′-TTTGAACAGGAAAATCTA-3′). PCR products were subjected to electrophoretic separation and visualized by staining with ethidium bromide.

2.4. Expression vectors

The open reading frame of the M-ABC2 cDNA was cloned into the mammalian expression vector pcDNA3 to generate pcDNA3-ABC. The region encoding the first 101 amino acids of M-ABC2 was cloned into pEGFP-N1 to generate pM2-EGFP. The bacterial glutathione *S*-transferase (GST) fusion expression vector, pGEX9C, consisted of the region encoding the C-terminal 40 amino acids of M-ABC2 cloned into pGEX2T vector. The pM1-EGFP vector, encoding mitochondrial-targeted green fluorescent protein (GFP) was generated by Hogue et al. [6].

2.5. Protein expression

Human ovarian SKOV3 cells were stably transfected with pcDNA3-ABC vector by selection in Geneticin/G418 (Gibco BRL). Transient transfections were performed using monkey Cos1 cells and the cells were utilized 20 h post-transfection in microscopy studies. The GST–ABC fusion protein was expressed in DH5α cells transformed with pGEX-9C.

2.6. Membrane preparations

Total membranes from cultured cells were prepared by Dounce homogenization of cells suspended in ST buffer (250 mM sucrose, 5 mM Tris pH 8, 2 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin and 1% aprotinin). After centrifugation at $4500\times g$ (10 min) total membrane fractions were collected by centrifugation at $100\,000\times g$ (60 min).

Total membrane preparations (10 μg) were subjected to digestion with *N*-glycosidase F (Boehringer Mannheim) as described by Childs et al. [13]. Parallel experiments using identically processed membrane fractions were examined for deglycosylation of a known glycoprotein as positive control.

2.7. Immunological methods

Purified GST–ABC fusion protein was injected into New Zealand white rabbits and immune serum was pre-absorbed with paraformaldehyde-fixed SKOV3 cells as described [13]. Western blot analysis was performed as described by Hogue et al. [6], as was immunofluorescence staining, except anti-rabbit ALEXA 568 IgG (Molecular Probes) was used.

3. Results

3.1. Characterization of a new human ABC cDNA

The EST clone 193990 was identified as a new human ABC gene by comparison of the conserved NBD motifs of known ABC proteins to the EST database. A corresponding full-length human 3857 bp cDNA, encoding a protein of 738 amino acids and 79.1 kDa, was isolated. The encoded protein has the characteristics of a half ABC transporter containing a predicted N-terminal TMD and conserved Walker A, Walker

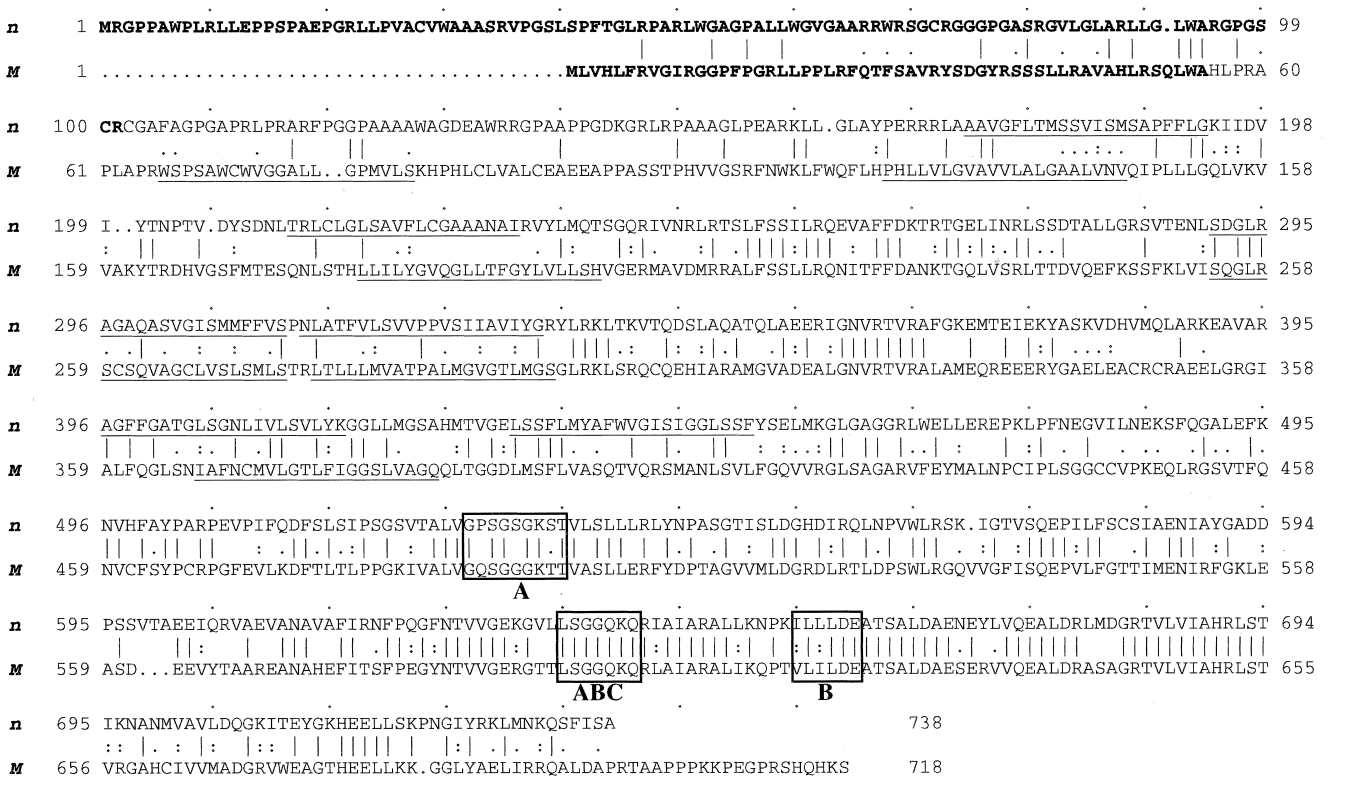

Fig. 1. Alignment of the new ABC (*n*) and M-ABC1 (*M*) proteins. The alignment was performed using the GAP program of the GCG package. Amino acids which have high or low similarity are marked by a colon or period, respectively. Walker A, Walker B and ABC signature motifs are boxed. Predicted transmembrane-spanning helices are underlined. The N-terminal sequences which are able to target GFP to mitochondria are in boldface.

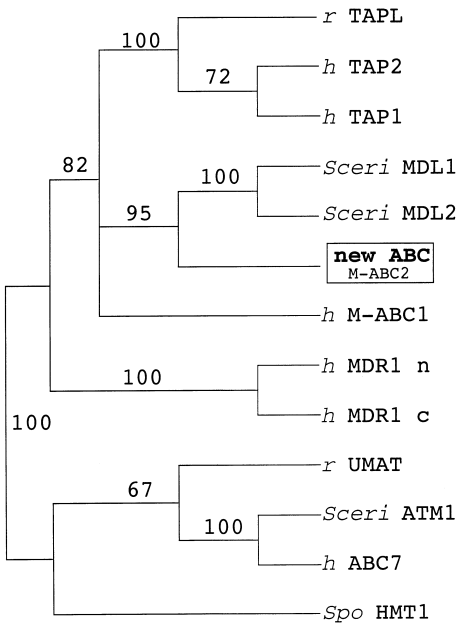

Fig. 2. Phylogenetic relationship of new ABC (M-ABC2) protein. Shown is a neighbor-joining bootstrap tree (values adjacent to each branch indicate the percentage of times each group was recovered in 500 replicates derived from amino acid sequences of representative ABC transporters within the same subgroup). MDR1 protein was split into the N-terminal half (n) and C-terminal half (c) in the analysis. (*h*, human; *r*, rat; *Sceri*, *S. cerevisiae*; *Spo*, *S. pombe*).

B and ABC signature motifs within the C-terminal region of the protein (Fig. 1).

Phylogenetic analysis of the new ABC protein, together with known ABC proteins, revealed that it falls into the same general cluster as P-glycoprotein, TAP1/2 and M-ABC1 and that it is most closely related to the MDL1 and MDL2 proteins of *S. cerevisiae* (Fig. 2). Further analysis using a pairwise comparison with all known human ABC proteins indicated that the new ABC protein is most similar to M-ABC1, the amino acid sequence alignment of which is shown in Fig. 1. The new ABC protein was therefore designated M-ABC2 based upon its close relationship to M-ABC1 and its subcellular localization as demonstrated within this study.

3.2. M-ABC2 pseudogene identification

The location of the M-ABC2 gene had previously been mapped, using a corresponding EST clone, to chromosome 1q42 by Allikmets et al. [9]. Interestingly, FISH analysis using our genomic HGL9C1 DNA clone also mapped the gene to chromosome 15q13–14 (Fig. 3A). Southern blot analysis of human genomic DNA revealed the existence of two copies of the M-ABC2 gene (not shown). PCR amplification of a monochromosomal somatic cell hybrid panel using primers specific to the new human ABC gene supported its location on both chromosomes 1 and 15 (not shown). Southern blot analysis revealed that only a single copy of the M-ABC2 gene exists within canine, swine and rhesus monkey genomes (not shown).

PCR amplification of the genomic HGL9C1 DNA clone, using primer sets encompassing 3000 bp of the M-ABC2 cDNA, generated an identical series of overlapping DNA fragments as that amplified from the M-ABC2 cDNA sequence (data not shown). This observation indicates that no introns exist within the genomic HGL9C1 DNA, suggesting that the M-ABC2 gene at chromosome 15q13–14 is a processed pseudogene. The genomic HGL9C1 and HGL9C3 clones were partially sequenced (corresponding to the M-ABC2 cDNA at positions 638–1090, 1406–1697 and 2441–3180 bp) and found to contain numerous nucleotide substitutions, deletions and insertions generating frame shifts and premature translational termination codons.

The ability of the chromosome 15-specific M-ABC2 gene to be transcribed was assessed by RT-PCR. Different oligonucleotide primers, specific for the 3′ untranslated regions of the M-ABC2 gene on either chromosome 1 or 15, were used in conjunction with an oligonucleotide primer common to both chromosome 1 and 15 genes. Analysis of the total RNA iso-

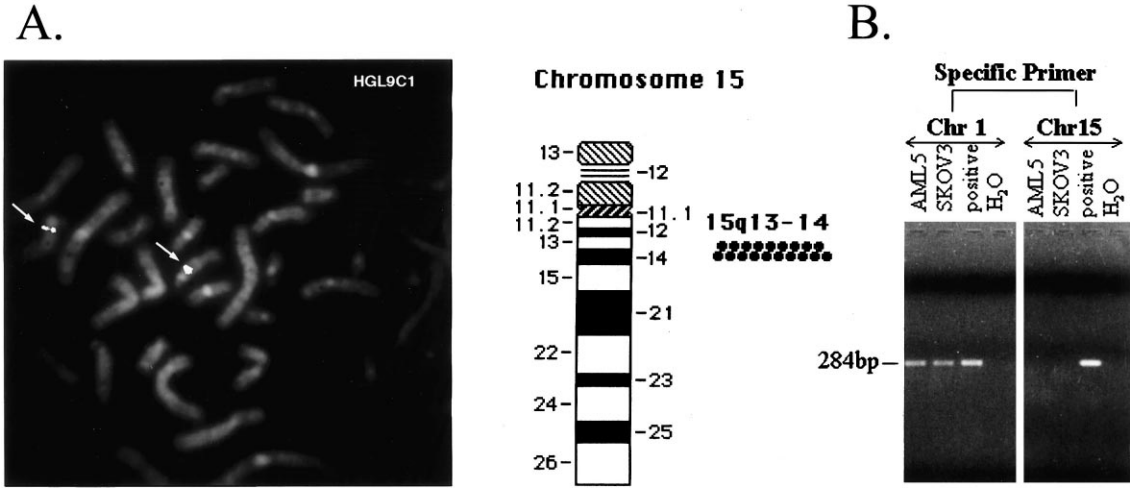

Fig. 3. Pseudogene identification. A: FISH analysis using genomic HGL9C1 DNA clone genomic mapped to chromosome 15q13–14. B: Analysis for alternate M-ABC2 gene expression. RT-PCR was used to detect different transcripts in RNA of cultured human AML5 (OCI/AML5, a human acute myeloid leukemia cell line established in Ontario Cancer Institute, Toronto, Canada) and SKOV3 cells expressed from the M-ABC2 genes on either chromosome 1 or chromosome 15. Positive controls: chromosome 1, M-ABC2 cDNA; chromosome 15, genomic HGL9C1 DNA.

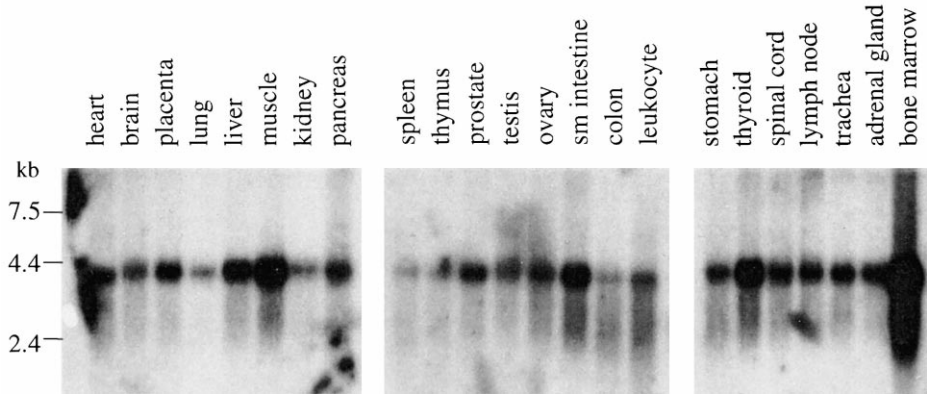

Fig. 4. Tissue distribution of the M-ABC2 transcript. Northern blot analysis was performed using human multiple tissue mRNA blots.

lated from human cell lines and human tissue revealed RT-PCR products to be generated only in the presence of the chromosome 1-specific primer (Fig. 3B). These results indicate that the chromosome 15-specific ABC gene is not expressed in the human cell lines and tissue examined. The pseudogene of M-ABC2 is a processed (intronless) pseudogene, different from the pseudogenes of other human ABC genes reported to date, which contain introns [14]. The biological significance, if any, of this pseudogene is not known.

3.3. mRNA tissue distribution

The pattern of human tissue expression for the M-ABC2 gene was examined by Northern blot analysis (Fig. 4). An approximate 4000 nucleotide mRNA species was observed in all human tissues; with highest expression levels in bone marrow, intermediate to high levels in skeletal muscle, small intestine, thyroid, heart, brain, placenta, liver, pancreas, prostate, testis, ovary, leukocyte, stomach, spinal cord, lymph node, trachea and adrenal gland, and low levels in lung, kidney, spleen, thymus and colon.

3.4. ABC protein expression

The expression of the M-ABC2 protein in cultured normal human SKOV3 and SKOV3 cells stably transfected with M-ABC2 cDNA (SK-ABC cells) was examined by Western blot analysis. An immunoreactive protein of approximately 60 kDa was detected by polyclonal antibody in membranes isolated from both cell types with, as would be expected, a higher level of expression in SK-ABC cells (Fig. 5A). Immunoreactivity towards the 60 kDa protein was specific, as the binding of antibody could be blocked by the presence of the GST–ABC fusion protein, but not by GST alone (Fig. 5A).

Two potential sites for Asn-linked glycosylation occur within the predicted amino acid sequence of the M-ABC2 protein. To examine the presence of Asn-linked oligosaccharide, isolated membranes were treated with *N*-glycosidase F. This treatment did not change the mobility of the immunoreactive band in membranes of SKOV3 and SK-ABC cells, indicating that the M-ABC2 protein was not subject to Asn-linked glycosylation.

3.5. Cellular localization

The close relatedness of the M-ABC2 protein with M-ABC1 suggested that the M-ABC2 was localized to mitochondria. To demonstrate cellular localization, Cos1 cells were transiently co-transfected with expression vectors encoding the M-ABC2 protein (pcDNA3-ABC) and mitochondrial-targeted GFP (pM1-EGFP) and subjected to immunofluorescence staining microscopy. The staining pattern for the M-ABC2-specific antibody in transfected cells overlapped closely with the subcellular distribution of mitochondrial-targeted GFP (Fig. 6A). These confirmed that the M-ABC2 protein resides within membranes of mammalian mitochondria.

Transient transfection of Cos1 cells with an expression vector encoding the first 101 amino acids of the M-ABC2 protein fused to GFP (pM2-EGFP) resulted in this GFP fusion protein being directed to subcellular compartments that co-stained with the mitochondrial-specific stain MitoTracker CMX-Ros (Fig. 6B). The presence of a mitochondrial-targeting signal within the extreme N-terminus region of M-ABC2 is consistent with that observed in other mitochondrial half ABC proteins [6,7,15].

4. Discussion

We have identified a new human half ABC protein that is most closely related to M-ABC1 and which also resides within membranes of mammalian mitochondria. Accordingly, we

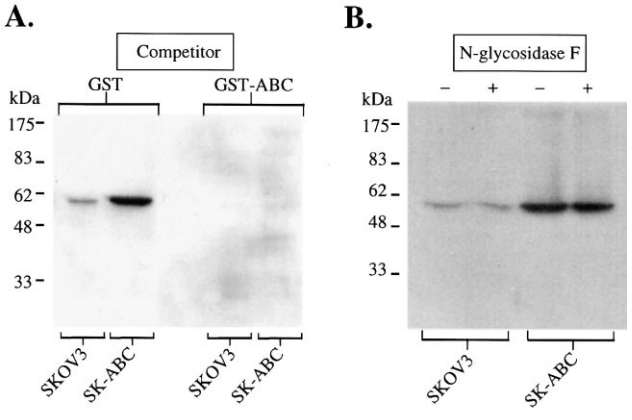

Fig. 5. Characterization of the M-ABC2 protein. A: Western blot analysis of membrane fractions of SKOV3 and SK-ABC cells using anti-ABC polyclonal serum pre-incubated with GST (GST), or GST–ABC fusion protein (GST–ABC). B: Western blot analysis using anti-M-ABC2 serum performed on membrane fractions treated with (+) or without (−) *N*-glycosidase F.

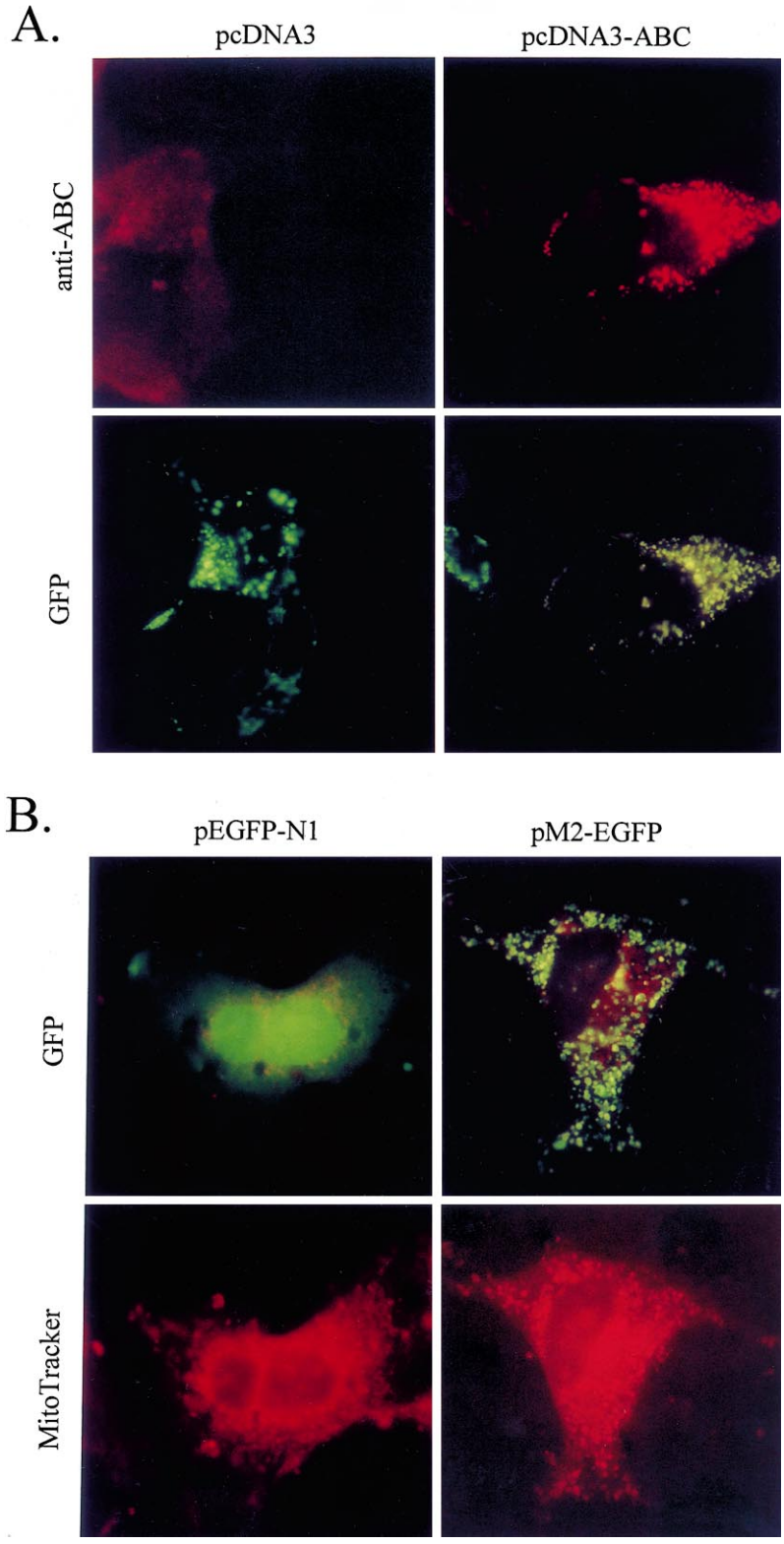

Fig. 6. Mitochondrial localization of the M-ABC2 protein. A: Cos1 cells were co-transfected with vectors expressing mitochondrial-targeted GFP (pM1-EGFP) and (i) no protein (pcDNA3) or (ii) the M-ABC2 protein (pcDNA3-ABC) and subjected to immunofluorescence staining. Shown are identical fields of cells examined for GFP fluorescence (GFP) or staining with anti-M-ABC2 serum (anti-ABC). B: Cos1 cells were transfected with vectors expressing GFP alone (pEGFP-N1) or the N-terminal 101 amino acids of the M-ABC2 protein fused to the N-terminus of GFP (pM2-EGFP) and then incubated with the mitochondria-specific marker, MitoTracker CMX-Ros. Shown is the fluorescence of GFP or MitoTracker for identical fields of cells.

have designated this protein as 'mitochondrial-ABC2' (M-ABC2).

Phylogenetic analysis indicated that both M-ABC1 and M-ABC2 are closely related to MDL1 and MDL2 of *S. cerevisiae*, two half ABC transporters whose functions or cellular locations are not yet defined [16]. Interestingly, the other identified mitochondrial ABC7 and ATM1 proteins fall within a phylogenetically distinct cluster along with the rat UMAT protein [17] and *Schizosaccharomyces pombe* HMT1, an ABC protein involved in heavy metal transport [18]. These relationships between known mitochondrial ABC proteins suggest that M-ABC1 and M-ABC2 perform functions distinct from that of ABC7 and ATM1. This prediction is supported by observations that the NBD of ATM1 resides within the mitochondrial matrix [15] and the NBD of M-ABC1 resides within the mitochondrial intermembrane space (D.L. Hogue, unpublished).

The close relatedness of M-ABC2 and M-ABC1 suggests that these proteins may exhibit overlapping function, as has been demonstrated for ABC7 and ATM1. In addition to previously described potential functions for mitochondrial ABC proteins [6], a candidate role for M-ABC1 and/or M-ABC2 may be the recently described nucleotide-gated channel for K^+ uptake into mitochondria [19], which is a functional equivalent of plasma membrane sulfonylurea receptor/K^+ channels that contain the full ABC protein, SUR [20].

All half ABC proteins seemingly require oligomerization into multimeric complexes to be functionally competent, as has been elegantly demonstrated for the heteromeric TAP1/TAP2 complex [21]. While it is tempting to speculate that closely related half ABC proteins, such as M-ABC1 and M-ABC2, are oligomerization partners, the ability of individual half ABC proteins to function as homomeric complexes cannot be excluded. Indeed, with the recent identification of at least 11 different human half ABC proteins [5,22], a major obstacle in the study of their functions will be delineating the identity of proteins that constitute a functional oligomeric complex.

In summary, this study has identified a third member of the mammalian mitochondrial ABC subfamily. In addition, our phylogenetic analysis predicts that the UMAT protein [17] may be a fourth member of this subfamily, which currently consists of M-ABC1, M-ABC2 and ABC7.

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