Identification of the human mitochondrial *S*-adenosylmethionine transporter: bacterial expression, reconstitution, functional characterization and tissue distribution

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The mitochondrial carriers are a family of transport proteins that, with a few exceptions, are found in the inner membranes of mitochondria. They shuttle metabolites and cofactors through this membrane, and connect cytoplasmic functions with others in the matrix. SAM (*S*-adenosylmethionine) has to be transported into the mitochondria where it is converted into *S*-adenosylhomocysteine in methylation reactions of DNA, RNA and proteins. The transport of SAM has been investigated in rat liver mitochondria, but no protein has ever been associated with this activity. By using information derived from the phylogenetically distant yeast mitochondrial carrier for SAM and from related human expressed sequence tags, a human cDNA sequence was completed. This sequence was overexpressed in bacteria, and its product was purified, reconstituted into phospholipid vesicles and identified from its transport properties as the human mitochondrial SAM carrier (SAMC). Unlike the yeast orthologue, SAMC catalysed virtually only countertransport, exhibited a higher transport affinity for SAM and was strongly inhibited by tannic acid and Bromocresol Purple. SAMC was found to be expressed in all human tissues examined and was localized to the mitochondria. The physiological role of SAMC is probably to exchange cytosolic SAM for mitochondrial *S*-adenosylhomocysteine. This is the first report describing the identification and characterization of the human SAMC and its gene.

Key words: mitochondria, mitochondrial carrier, proteomics, *S*-adenosylmethionine carrier, transport.

INTRODUCTION

SAM (*S*-adenosylmethionine) is the methyl group donor for almost all biological methylation reactions. In mitochondria, it is required for the methylation of DNA, RNA and proteins [1–20] and as an intermediate in the biosynthesis of lipoic acid [21,22], ubiquinone [23,24] and, in yeast, biotin [25,26]. Since mitochondria have a relatively large pool of SAM [27] and the enzyme necessary for its synthesis, methionine adenosyltransferase, is present in the cytosol and not in the mitochondria (see [28] and references therein), it was hypothesized that a carrier-mediated system is responsible for the transport of SAM from the cytosol into the mitochondria. The biochemical properties of this system have indeed been investigated in rat liver mitochondria by Horne et al. [28]. It was found that the uptake of SAM into mitochondria is saturable, displays countertransport and is inhibited by the close SAM structural analogues SAHC (*S*-adenosylhomocysteine) and adenosylornithine. However, the protein responsible for this transport has not hitherto been identified in mammals.

In the present study, the identification and characterization of the human mitochondrial SAMC (*S*-adenosylmethionine carrier) is described. By using a *Saccharomyces cerevisiae* protein, Sam5p (*S. cerevisiae* SAMC protein), which has very recently been shown in our laboratory to be a SAM transporter [29], human ESTs (expressed sequence tags) were identified that encode fragments of a related protein. They provided information to complete the human cDNA sequence. The encoded protein was 274 amino acids long, had the characteristic features of the mitochondrial carrier family (see [30] for a review) and was found to be localized to mitochondria. It was overexpressed in *Escherichia coli*, purified, reconstituted into phospholipid vesicles and shown from its transport and kinetic properties to be the human mitochondrial transporter for SAM. The main function of the protein is probably to catalyse the import of SAM into mitochondria in exchange for intramitochondrial SAHC produced from SAM in the methylation reactions. This paper presents the first information on the molecular properties of the human mitochondrial carrier for SAM and a definitive identification of its gene.

EXPERIMENTAL

Sequence search and analysis

Human EST databases (www.ebi.ac.uk, ensembl.ebi.ac.uk and www.ncbi.nlm.nih.gov) were screened with the sequence of Sam5p [29] using TBLASTN. The ESTs were assembled by SeqMan (Lasergene). Human genomic databases were screened with SAMC. The amino acid sequences were aligned with ClustalW (version 1.7). The phylogenetic tree was constructed

Abbreviations used: BFP, blue fluorescent protein; CAC, carnitine-acylcarnitine carrier; CHO, Chinese-hamster ovary; EST, expressed sequence tag; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; SAC, *S*-adenosylcysteine; SAHC, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SAMC, *S*-adenosylmethionine carrier; Sam5p, *Saccharomyces cerevisiae* SAMC protein.

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The nucleotide sequence data reported for SAMC will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ580932. The name SLC25A26 has been reserved for the gene encoding SAMC.

by the neighbour-joining method [31] using the MacVector 7.2 software (Accelrys, Cambridge, U.K.) and the PAM250 matrix. The phylogenetic distances were calculated automatically as *P* values; $P = 0.1$ corresponds approximately to a difference of 10 % between two sequences.

Bacterial expression and purification of SAMC

The coding sequence for SAMC was amplified by PCR from human brain cDNA (ClonTech, Palo Alto, CA, U.S.A.). The oligonucleotide primers corresponding to the extremities of the coding sequence were synthesized (accession no. AJ580932), with additional *Nde*I and *Eco*RI sites. The product was cloned into the pRUN expression vector (derived from pKN172 [32]), and the construct was transformed into *E. coli* TOP 10 cells (Invitrogen). Transformants were selected on 2× TY plates containing ampicillin (100 μg/ml) and screened by direct colony PCR and restriction digestion of plasmids. Overproduction of SAMC as inclusion bodies in the cytosol of *E. coli* was accomplished as described before [32], except that the host cells were *E. coli* BL-21 CodonPlus(DE3)-RIL (Stratagene). Control cultures with the empty vector were processed in parallel. Inclusion bodies were isolated and SAMC was purified by centrifugation and washing steps as described previously [33–35].

Reconstitution into liposomes and transport assays

The recombinant protein in sarkosyl was reconstituted into liposomes in the presence or absence of substrates, as described before [36]. External substrate was removed from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with 50 mM NaCl and 10 mM Pipes at pH 7.0 (buffer A). Transport at 25 °C was started by adding [^{3}H]SAM (NEN) to substrate-loaded proteoliposomes (exchange) or to empty proteoliposomes (uniport). In both cases, transport was terminated by the addition of 20 mM pyridoxal 5′-phosphate and 2 mM *p*-hydroxymercuribenzoate (the 'inhibitor-stop' method [36]). In controls, the inhibitors were added at the beginning together with the radioactive substrate. All transport measurements were performed at the same internal and external pH values (10 mM Pipes, pH 7.0). Finally, the external substrate was removed and the radioactivity in the liposomes was measured [36]. The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the radioactivity of proteoliposomes after 45 s (in the initial linear range of substrate uptake). Alternatively, the initial transport rate was calculated from the time course of isotope equilibration [36]. The reconstituted protein was also assayed for other transport activities. For efflux measurements, proteoliposomes containing 1 mM SAM were labelled with 5 μM [^{3}H]SAM by carrier-mediated exchange equilibration [36]. After 60 min, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75. Efflux was started by adding unlabelled external substrate or buffer A alone, and terminated by adding the inhibitors indicated above.

Expression analysis by real-time PCR

Total RNAs from human tissues (Invitrogen) were reverse-transcribed with the GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA, U.S.A.) with random hexamers as primers. For real-time PCRs, the primers and the probe, based on the SAMC cDNA sequence (accession no. AJ580932), were designed with Primer Express (Applied Biosystems). The forward and reverse primers corresponded to nt 346–366 and 409–432 respectively, and the FAM-Dark Quencher labelled probe to nt 369–397 of the SAMC cDNA sequence. Real-time PCRs were performed in a MicroAmp optical 96-well plate using the automated ABI Prism 7000 Sequence Detector System (Applied Biosystems). The reaction mixture (50 μl) contained template (reverse-transcribed first-stranded cDNA; 5 μl), 1× TaqMan Universal Master Mix (Applied Biosystems), 200 nM probe for SAMC and 900 nM of each primer. To correct for differences in the amount of starting first-stranded cDNAs, the human β-actin gene was amplified in parallel as a reference endogenous housekeeping gene. The relative quantification of SAMC in various tissues was performed according to the comparative method ($2^{-\Delta\Delta Ct}$; [37,38] and Applied Biosystems User Bulletin no. 2P/N 4303859), with the liver ΔCt as internal calibrator. We have $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct(sample) - \Delta Ct(calibrator)]}$, where $\Delta Ct(sample) = Ct(sample) - Ct(reference\ gene)$ and Ct stands for the threshold cycle, i.e. the PCR cycle number at which emitted fluorescence exceeds 10 S.D. of baseline emissions. For the internal calibrator, $\Delta\Delta Ct = 0$ and $2^0 = 1$. For the remaining tissues, the value of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator (liver).

Subcellular localization

For subcellular localization of SAMC in CHO (Chinese-hamster ovary) cells, the protein was fused to EGFP (enhanced green fluorescent protein) at the C-terminus. For this purpose, the SAMC coding sequence was amplified without the termination codon and with additional *Hin*dIII and *Kpn*I restriction sites. The PCR product was cloned into a modified pcDNA3 vector [39] in frame with the HA1-EGFP sequence (where HA1 is a 9-amino-acid epitope derived from haemagglutinin). CHO cells were grown on 24 mm coverslips to 50–70 % confluence and co-transfected according to a standard calcium phosphate procedure with 4 μg of mtEBFP/pcDNA1 [40] (where BFP stands for blue fluorescent protein) and 4 μg of the modified pcDNA3 plasmid containing the coding sequence of SAMC fused with the EGFP sequence. EGFP and EBFP fluorescence were detected as described in [40]. In some experiments, CHO cells were transfected only with the SAMC–EGFP fusion construct. Then, they were incubated for 30 min at 37 °C in the presence of 200 nM MitoTracker Red CMXRos and washed twice with PBS. The MitoTracker Red fluorescence was detected as described in [29], except that the microscope was equipped with a Plan-Neofluar 63× oil objective.

Other methods

Proteins were analysed by SDS/PAGE and stained with Coomassie Blue dye. N-terminal sequencing was performed as described before [41]. The amount of pure SAMC was estimated by laser densitometry of stained samples, using carbonic anhydrase as protein standard [41]. The amount of protein incorporated into liposomes was measured as described in [41], and varied between 18 and 25 % of the protein added to the reconstitution mixture.

RESULTS

Isolation and characterization of the human SAMC cDNA

By screening human EST databases with the yeast Sam5p sequence [29], some related partial human ESTs were found. These

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SAMC   MDRPGFVAALVAGGVAGVSVDLILFPLDTIKTRLQSPQGFNKAGGFHGIYAGVPSAAIGS
Sam5p  MN--TFFLSLLSGAAAGTSTDLVFFPIDTIKTRLQAKGGFFANGGYKGIYRGLGSAVVAS
       *    *   *  *  ** * **  ** ********   **   **  *** *  **   *

SAMC   FPNAAAFFITYEYVKW--------FLHADSSSYLTPMKHMLAASAGEVVACLIRVPSEVV
Sam5p  APGASLFFISYDYMKVKSRPYISKLYSQGSEQLIDTTTHMLSSSIGEICACLVRVPAEVV
        * *  *** * * *              *        ***  * **  *** *** ***

SAMC   KQRAQVSASTRTFQIFSNILY---EEGIQG-LYRGYKSTVLREIPFSLVQFPLWESLKAL
Sam5p  KQRTQVHSTNSSWQTLQSILRNDNKEGLRKNLYRGWSTTIMREIPFTCIQFPLYEYLKKT
       *** **       *    **     **    ****   *  *****   **** * **

SAMC   WSWR-QDHVVDSWQSAVCGAFAGGFAAAVTTPLDVAKTRITLAKAGSSTADGNVLSVLHG
Sam5p  WAKANGQSQVEPWKGAICGSIAGGIAAATTTPLDFLKTRLMLNKTTASLG-----SVIIR
       *        *  *  * **  *** *** *****  ***  * *   *       **

SAMC   VWRSQGLAGLFAGVFPRMAAISLGGFIFLGAYDRTHSLLLEVGRKSP
Sam5p  IYREEGPAVFFSGVGPRTMWISAGGAIFLGMYETVHSLLSKSFPTAGEMRA
         *  * *  * ** **   ** ** **** *   ****
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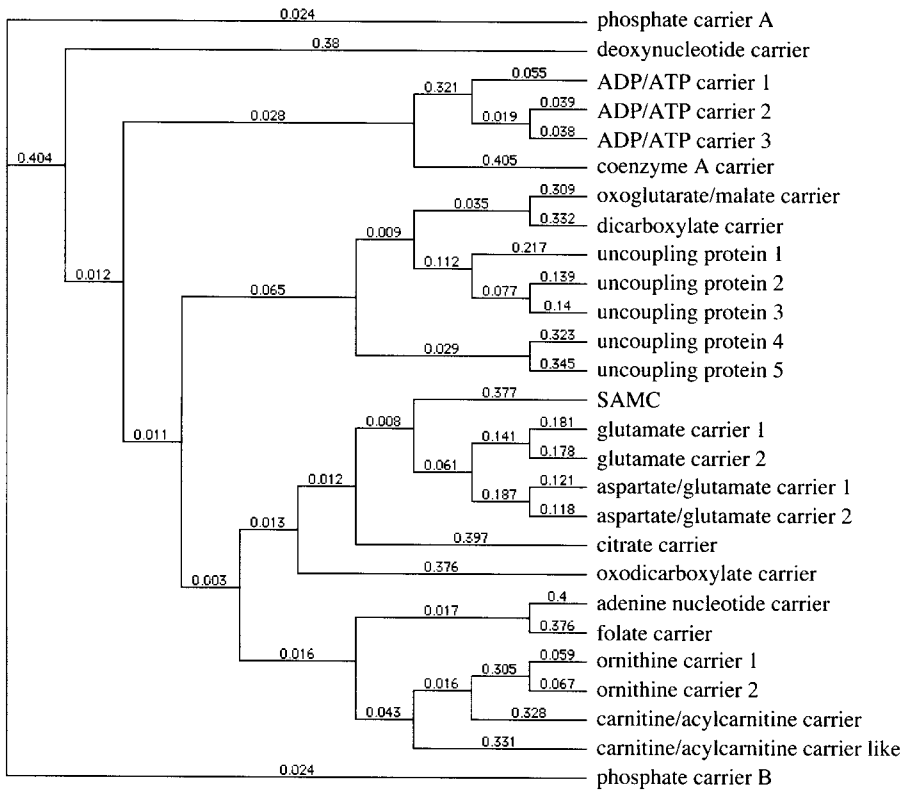

Figure 1 A novel human mitochondrial carrier protein SAMC

Upper panel: amino acid sequence alignment of SAMC with the *S. cerevisiae* Sam5p. Lower panel: unrooted phylogenetic tree of the human mitochondrial carriers of known function. Phylogenetic distances are indicated by the numbers reported in the Figure; a $P = 0.1$ corresponds approximately to a difference of 10 % between two sequences.

sequences were assembled, the contig was extended to the 5′- and 3′-ends and a full-length cDNA was obtained and sequenced. The final cDNA (accession no. AJ580932) consists of 1189 nucleotides. The ATG codon at nt 76–78 is preceded by an in-frame stop codon 9 bp upstream and is probably the translational-initiation codon. The open reading frame encodes a polypeptide of 274 amino acids (Figure 1, upper panel) with no targeting presequence, a pI of 9.51 and a molecular mass of 29.378 kDa. In a phylogenetic tree of the human mitochondrial carriers of known function (reviewed in [30]), SAMC is positioned close to the cluster that groups transporters for amino acids, i.e. the isoforms of the glutamate and the aspartate/glutamate carriers (Figure 1, lower panel). The human genome contains a single gene for SAMC and there are no isoforms with closely related gene sequences. The SAMC gene (named SLC25A26) is spread over at least 135 kb of the human genome, contains nine exons separated by eight introns and is localized on chromosome 3p14.3.

Expression of the mRNAs for human SAMC in various tissues

The tissue distribution of mRNAs for the human SAMC determined by real-time PCR is summarized in Figure 2. The amount of the SAMC mRNA in testis was severalfold higher than that present in liver, which served as an internal calibrator in the relative quantification of the SAMC mRNAs in various tissues. The SAMC mRNA was expressed in brain, heart, kidney, lung, skeletal muscle, pancreas and small intestine at levels comparable with those present in liver. Lower levels were detected in spleen. It should be noted that, as post-transcriptional mechanisms may

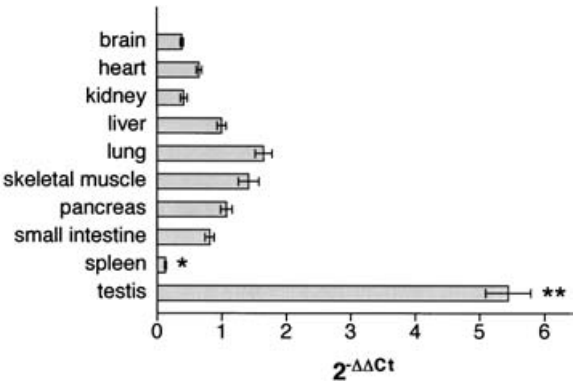

Figure 2 Expression of human SAMC in various tissues

Real-time PCR experiments were conducted on cDNAs prepared by reverse transcription of total RNAs from various human tissues, using specific primers and a probe based on human SAMC cDNA. The values $2^{-\Delta\Delta Ct}$ on the abscissa, which indicate the fold change in gene expression relative to the internal calibrator (liver), were calculated as described in the Experimental section. The data are means ± S.E.M. from four replicates. The significance of the differences in gene expression between testis and liver and between spleen and liver is indicated (**$P < 0.001$ and *$P < 0.01$ respectively, one-way ANOVA followed by Bonferroni *t*-test).

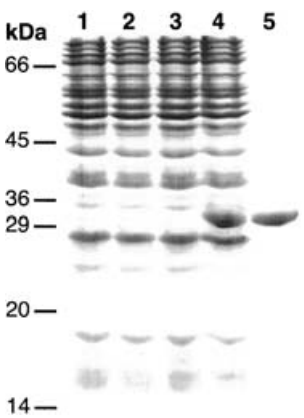

Figure 3 Overexpression in *E. coli* and purification of human SAMC

Proteins were separated by SDS/PAGE and stained with Coomassie Blue. Markers on the left-hand side column (ovotransferrin, BSA, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c*); lanes 1–4, *E. coli* BL-21 CodonPlus(DE3)-RIL containing the expression vector, without (lanes 1 and 3) and with the coding sequence of SAMC (lanes 2 and 4). Samples were taken at the time of induction (lanes 1 and 2) and 5 h later (lanes 3 and 4). The same number of bacteria was analysed in each sample. Lane 5, purified SAMC protein (4 μg) originating from bacteria shown in lane 4.

operate, these levels do not necessarily reflect the ratios of transport activities.

Bacterial expression of the human SAMC

The human SAMC was overexpressed in *E. coli* BL-21 CodonPlus(DE3)-RIL (see Figure 3, lane 4) in the form of inclusion bodies. The purified protein gave a single band by SDS/PAGE (Figure 3, lane 5) with an apparent molecular mass of 30.2 kDa (calculated value with initiator methionine, 29.4 kDa). Its identity was confirmed by N-terminal sequencing. The protein was not detected in bacteria harvested immediately before induction of expression (Figure 3, lanes 1 and 2), nor in cells harvested after induction but lacking the coding sequence in the expression vector (lane 3). Approx. 30 mg of purified protein was obtained per litre of culture.

Table 1 Dependence of the transport properties of SAMC on internal substrate

Proteoliposomes were preloaded internally with various substrates (concentration, 10 mM). Transport was started by adding 23 μM [^{3}H]SAM to proteoliposomes reconstituted with SAMC and terminated after 45 s. Data are means ± S.E.M. for at least three independent experiments. The significance of the difference between samples preloaded internally with SAM or SAHC and with no substrate (NaCl present) is indicated (*$P < 0.001$, one-way ANOVA followed by Bonferroni *t* test).

Internal substrate	[^{3}H]SAM transport [nmol · min^{-1} · (mg of protein)$^{-1}$]
None (NaCl present)	8 ± 2
SAM	284 ± 45*
SAHC	158 ± 29*
SAC	91 ± 20
Adenosylornithine	75 ± 14
Methionine	10 ± 2
5′-Deoxyadenosine	7 ± 2
5′-Methylthioadenosine	6 ± 2
AMP	12 ± 3
Ornithine	9 ± 2
Carnitine	10 ± 2
Choline	5 ± 2

Functional characterization of human SAMC

Proteoliposomes reconstituted with recombinant SAMC catalysed an exchange of external [^{3}H]SAM for internal SAM, which was inhibited completely by a mixture of pyridoxal 5′-phosphate and *p*-hydroxymercuribenzoate. They did not catalyse homoexchanges for phosphate, carnitine, glutamate, aspartate, glutamine, methionine, ornithine, L-malate, AMP and ADP (internal concentration, 10 mM; external concentration, 1 mM). Importantly, no [^{3}H]SAM/SAM exchange activity was detected if SAMC had been boiled before incorporation into liposomes nor by reconstitution of sarkosyl-solubilized material from bacterial cells either lacking the expression vector for SAMC or harvested immediately before induction of expression.

The substrate specificity of human SAMC was investigated in greater detail by measuring the uptake of [^{3}H]SAM into proteoliposomes that had been preloaded with a variety of substrates (Table 1). The highest activity of [^{3}H]SAM uptake into proteoliposomes was with internal SAM. [^{3}H]SAM also exchanged significantly with internal SAHC and, to a lesser extent, with internal SAC (*S*-adenosylcysteine) and adenosylornithine. In contrast, a very low uptake of labelled substrate was observed in the presence of internal methionine, 5′-deoxyadenosine, 5′-methylthioadenosine, AMP, ornithine, carnitine, choline and (results not shown) adenosine, betaine, ADP, thiamine, thiamine pyrophosphate, NMN, FMN, NAD, FAD, CoA, oxoglutarate, citrate, cysteine, glutamate, glutamine, lysine, arginine and serine. Reconstituted SAMC, therefore, displays a very narrow substrate specificity, which is confined only to SAM and its closest analogues.

The [^{3}H]SAM/SAM exchange reaction catalysed by reconstituted SAMC was inhibited strongly by pyridoxal 5′-phosphate, *p*-hydroxymercuribenzoate, mersalyl and mercuric chloride (inhibitors of several mitochondrial carriers) as well as by tannic acid and Bromocresol Purple, which are specific inhibitors of the glutamate carrier [38] (see Figure 4). In contrast, carboxyatractyloside, bongkrekate, *N*-ethylmaleimide, α-cyano-4-hydroxycinnamate, butylmalonate and 1,2,3-benzenetricarboxylate, inhibitors of other characterized mitochondrial carriers, had little or no effect on the activity of reconstituted SAMC. Similarly, little effect was exerted by bathophenanthroline, which inhibits

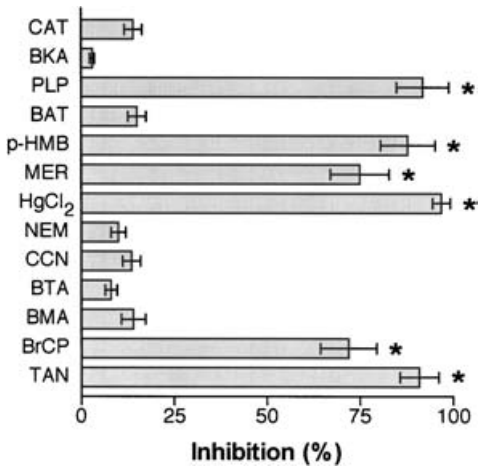

Figure 4 Effect of inhibitors on the [^{3}H]SAM/SAM exchange by SAMC

Proteoliposomes were preloaded internally with 10 mM SAM and transport was initiated by adding 23 μM [^{3}H]SAM to proteoliposomes reconstituted with SAMC. The incubation time was 45 s. The inhibitors were added 3 min before the labelled substrate. The final concentrations of the inhibitors were 10 μM for CAT (carboxyatractyloside) and BKA (bonkrekic acid), 20 mM for PLP (pyridoxal 5′-phosphate) and BAT (bathophenanthroline), 200 μM for p-HMB (*p*-hydroxymercuribenzoate) and MER (mersalyl) and $HgCl_2$, 1 mM NEM (*N*-ethylmaleimide) and CCN (α-cyanocinnamate), 2 mM BMA (butylmalonate) and BTA (1,2,3-benzenetricarboxylate), 0.1 % TAN (tannic acid) and 0.1 mM BrCP (Bromocresol Purple). Data are means $\pm$ S.E.M. for at least three experiments. The significance of the difference between the extents of inhibition by PLP, p-HMB, MER, $HgCl_2$, BrCP and TAN and by the other compounds is indicated (*$P < 0.001$, one-way ANOVA followed by Bonferroni *t* test).

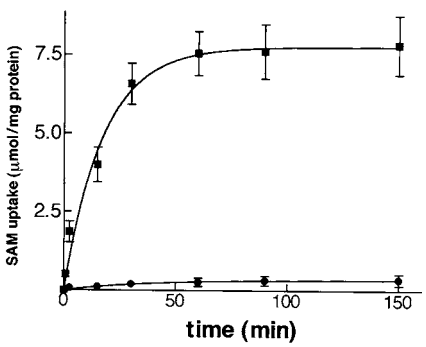

Figure 5 Kinetics of [^{3}H]SAM transport in proteoliposomes reconstituted with SAMC

1 mM [^{3}H]SAM was added to proteoliposomes containing 10 mM SAM (exchange, ■) or 10 mM NaCl and no substrate (uniport, ●). Data are means $\pm$ S.E.M. for three independent experiments.

several mitochondrial carriers. Furthermore, the uptake of 23 μM [^{3}H]SAM by proteoliposomes containing 10 mM SAM (reaction time 45 s) was inhibited by external addition of 230 μM SAHC (94 % inhibition), SAC (70 %) or adenosylornithine (29 %). In contrast, a very low inhibition, if any, was detected with external 230 μM adenosine, 5′-methylthioadenosine, 5′-deoxyadenosine, methionine, cysteine, ornithine, carnitine, AMP, NMN or glutamate (results not shown).

Kinetic characteristics of human SAMC

In Figure 5, the kinetics are compared for the uptake by proteoliposomes of 1 mM [^{3}H]SAM measured either as uniport (in the absence of internal SAM) or as exchange (in the presence of 10 mM SAM). The uptake of SAM by exchange followed first-order kinetics [rate constant, 0.06 min^{-1}; initial rate, 442 nmol · min^{-1} · (mg of protein)$^{-1}$], isotopic equilibrium being approached exponentially. In contrast, the uniport uptake of SAM was very low. In agreement with these results, a substantial efflux of [^{3}H]SAM from prelabelled proteoliposomes was observed in the presence of external 1 mM SAM or SAHC, whereas the efflux was negligible without added substrate (results not shown).

The kinetic constants of the recombinant purified SAMC were determined by measuring the initial transport rate at various external [^{3}H]SAM concentrations, in the presence of a constant saturating internal concentration of 10 mM SAM. The transport affinity (K_m) and the specific activity (V_{max}) values for SAM/SAM exchange at 25 °C were 23.0 $\pm$ 2.5 μM and 463 $\pm$ 95 nmol · min^{-1} · (mg of protein)$^{-1}$, respectively, in 18 experiments. SAHC, SAC and adenosylornithine inhibited [^{3}H]SAM/SAM exchange competitively by increasing the apparent K_m without changing the V_{max} of SAM uptake (results not shown). The inhibition constants (K_i) of SAHC, SAC and adenosylornithine were 10.5 $\pm$ 1.4, 44.8 $\pm$ 5.7 and 287.2 $\pm$ 20.9 μM respectively (in at least three experiments for each inhibitor).

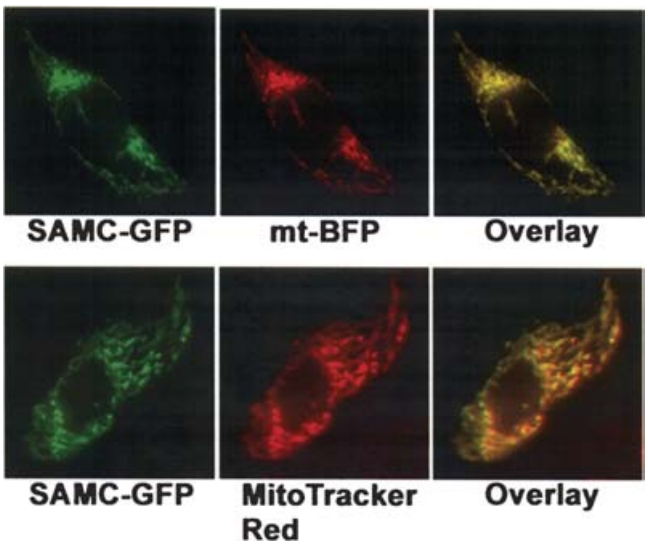

Figure 6 Subcellular localization of human SAMC

Upper panel: CHO cells were transiently co-transfected with pcDNA3 vector carrying the DNA sequence coding SAMC in-frame with GFP DNA sequence and pcDNA1 plasmid carrying the DNA sequence of mitochondrially targeted BFP. Lower panel: CHO cells were transfected with the SAMC-GFP construct and MitoTracker Red was used to locate mitochondria in the cells. SAMC-GFP, fluorescence of GFP fused to SAMC; mt-BFP, fluorescence of the mtBFP (here shown in red); MitoTracker Red, fluorescence of MitoTracker Red; Overlay, merged image of mtBFP fluorescence (upper panel) or MitoTracker Red fluorescence (lower panel) with SAMC-GFP fluorescence. Images were acquired by a fluorescence microscope equipped with appropriate filters, and identical fields are presented.

Subcellular localization of SAMC

Since some members of the mitochondrial carrier family are localized in non-mitochondrial membranes [42–44], the intracellular localization of SAMC was investigated. This aspect was addressed by constructing an expression plasmid with GFP fused to SAMC (see Figure 6). CHO cells were transfected with the pcDNA3-SAMC-EGFP plasmid, and images were acquired

36–48 h after transfection. Approx. 30–40 % of cells were transfected, and the green fluorescence revealed a typical mitochondrial localization of SAMC. The green fluorescence of the GFP-tagged protein completely overlapped with the blue fluorescence of a mitochondrially targeted BFP [39] co-expressed in the same cells (Figure 6, upper panel). Similarly, with the mitochondrial-specific dye, MitoTracker Red, CHO cells expressing the GFP-tagged SAMC showed a pattern of fluorescence that coincides with that of the mitochondria (Figure 6, lower panel). Thus SAMC, which lacks the targeting N-terminal extension, contains in its amino acid sequence the structural information for its import into the mitochondria, in accordance with data available for other mitochondrial carriers [45,46].

DISCUSSION

SAMC (encoded by the SLC25A26 gene) belongs to the mitochondrial carrier family of proteins, which have different functions but related sequences. Drawing conclusions about the identification of a mitochondrial carrier of unknown function only on the basis of a reasonably high identity with a member of the family functionally identified in another organism, can be misleading for two reasons. First, there is a significant homology between different members of the mitochondrial carrier family; secondly, the structural determinants of the specific transport function catalysed by an individual member in various organisms are not yet known. For example, the novel plant dicarboxylate–tricarboxylate carrier [34], which displays 41, 29 and 20 % identity with the human oxoglutarate, dicarboxylate and citrate carriers respectively, and which was previously reported to be an oxoglutarate carrier [34,47], in fact transports citrate (tricarboxylates) and oxoglutarate with high efficiency, and, to a lesser extent, malate but not phosphate (malate and phosphate are the best substrates of the dicarboxylate carrier). Furthermore, the closest *S. cerevisiae* sequence YLR348c to the bovine oxoglutarate carrier (37 % identity) was found not to transport oxoglutarate, but malate and phosphate being the yeast dicarboxylate carrier [48]. In addition, the two human isoforms of the ornithine carrier (87 % identity) differ considerably in their substrate specificity [35], whereas the recently identified human CACL [carnitine-acylcarnitine carrier-like; 33 % identical with the known CAC (carnitine-acylcarnitine carrier)] isoform shares with CAC the ability to transport acylcarnitine esters efficiently [49,50]. In the present study, we have expressed the SAMC cDNA in *E. coli* and have functionally characterized the purified SAMC after reconstitution into phospholipid vesicles. The same strategy has in recent years enabled the definitive identification and detailed characterization of several novel mitochondrial transporters (see [30,51,52] for reviews).

From the transport properties and kinetic characteristics, as well as the subcellular localization of SAMC, reported here, it can be concluded that this protein is the human mitochondrial transporter for SAM. As yet, there is no definite proof that it is closely related to the carrier for SAM detected in rat liver mitochondria [28], which has not been analysed by protein sequencing, although it is probably so. The biochemical properties of the recombinant reconstituted SAMC are similar to those of the yeast Sam5p [29]. However, SAMC differs markedly from the yeast orthologue in several respects. The main differences are (i) the almost complete inability of the human protein to catalyse uniport, at variance with Sam5p, (ii) the higher transport affinity of the human protein for SAM and (iii) the strong sensitivity of the human protein to tannic acid and Bromocresol Purple, which are known inhibitors of the glutamate carrier [38] and do not affect the activity of Sam5p [29]. Interestingly, the behaviours of SAMC and Sam5p towards tannic acid and Bromocresol Purple correlate with the observations that in phylogenetic trees of mitochondrial carriers, SAMC clusters together with transporters for amino acids (Figure 1, lower panel), whereas Sam5p (YNL003c) clusters together with transporters for phosphate, thiamine pyrophosphate (YGR096w) and nucleotides [53]. In view of the identification of SAMC reported here, and of its phylogenetically distant yeast Sam5p [29] as mitochondrial carriers for SAM, several protein sequences available in databases are probably orthologues of these transporters in other organisms. These sequences include Q8JZT2 from *Mus musculus*, Z68160 from *C. elegans*, Q9VBN7 from *D. melanogaster*, EAA08873 from *A. gambie* and EAA26969 from *N. crassa*.

As SAM is produced in the cytosol and is required in mitochondria, the primary function of SAMC is to catalyse the uptake of SAM into mitochondria. However, since SAMC functions almost exclusively by a counter-exchange mechanism, the carrier-mediated uptake of SAM requires the efflux of a counter-substrate. On the basis of transport measurements, SAHC produced from SAM in the methylation reactions and hydrolysed exclusively in the cytosol [29] may serve as the counter-substrate of SAMC for SAM. Therefore the physiological role of the human SAMC is most probably to catalyse the uptake of SAM into the mitochondrial matrix in exchange for internal SAHC.

SAMC is crucial for mitochondrial metabolism, especially for the mitochondrial synthesis of proteins. SAM is in fact essential for the methylation of various types of nucleic acid present in the mitochondria. In mammals, several methylations of DNA [1–4], transfer RNA [6–10] and rRNA [11–14] have been identified, and some of them have been shown to be essential for function [6,7,16,17]. Besides being crucial for mitochondrial protein synthesis, SAM is necessary for post-translational modifications of some mitochondrial proteins. For example, prohibitin1 (PHB1) must be methylated to be stabilized and to function as a chaperone-like protein in the correct folding and assembly of subunits of the mitochondrial respiratory-chain complexes [18–20]. Interestingly, studies on knockout mice deficient in hepatic SAM synthetase (*MAT1A*$^{-/-}$), and in cultured rat hepatocytes using a medium without methionine or in the presence of cycloleucine, a known inhibitor of MAT1 activity, have shown a post-translational downregulation of prohibitin (PHB1) and of subunits I and II of cytochrome *c* oxidase without any decrease in their mRNA transcripts [20]. Furthermore, it has been shown that the decrease in PHB1 levels correlates with a loss of mitochondrial function [20]. In agreement with its importance in mitochondrial metabolism, SAMC is expressed in all human tissues that were analysed and appears to be widespread in eukaryotes. Furthermore, the *S. cerevisiae* mutant lacking the *SAM5* (YNL003c) gene (orthologue of the human SLC25A26) has long been known to cause a petite ρ^+ phenotype [29], which demonstrates the indispensable role of the mitochondrial SAM carrier for the growth of yeast cells on respiratory substrates. Interestingly, we have recently shown that this phenotype is rescued by expressing the cytosolic SAM synthetase1 (Sam1p) inside mitochondria [29].

In recent years, the cloning and functional identification of SLC25 genes has allowed us (i) to identify the genes (and their defects) responsible for some diseases, e.g. Stanley syndrome and Amish microcephaly, and (ii) where the genes were already known, to characterize the function of the gene products and hence understand the molecular basis and the symptoms of the diseases, e.g. type II citrullinaemia and HHH syndrome (see [30] for a review). It is probable that the identification and functional characterization of SAMC will lead to the elucidation of the

molecular basis of mitochondrial diseases caused by defects in the SLC25A26 gene.

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