Surface translocation and tri-iodothyronine uptake of mutant MCT8 proteins are cell type-dependent

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Abstract

Mutations in the gene encoding the thyroid hormone transporter, monocarboxylate transporter 8 (MCT8), underlie severe mental retardation. We wanted to understand the functional consequences of a series of missense mutations in MCT8 in order to identify therapeutic options for affected patients. We established cell lines stably expressing 12 MCT8 variants in JEG1 and MDCK1 cells. The cell lines were characterized according to MCT8 mRNA and protein expression, tri-iodothyronine (T_3) transport activity, substrate K_M characteristics, surface expression, and responsiveness to T_3 preincubation and chemical chaperones. Functional activities of ins235V and L568P MCT8 mutants depend on the cell type in which they are expressed. These mutants and R271H exhibited considerable transport activity when present at the cell surface as verified by surface biotinylation and kinetic analysis. Most mutants, however, were inactive in T_3 transport even when present at the cell surface (e.g. S194F, A224V, ΔF230, L512P). Preincubation of G558D with T_3 increased T_3 uptake in MDCK1 cells to a small, but significant, extent. Chemical chaperones were ineffective. The finding that the cell type determines surface expression and T_3 transport activities of missense mutants in MCT8 may be important to understand phenotypic variability among carriers of different mutations. In particular, the clinical observation that the severity of derangements of thyroid hormone levels does not correlate with mental impairments of the patients may be based on different residual activity of mutant MCT8 in different cell types.

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Introduction

Mutations affecting the gene encoding monocarboxylate transporter 8 (*MCT8*; *SLC16A2*) have been identified in patients suffering from a syndrome associated with X-linked mental retardation and with elevated serum tri-iodothyronine (T_3; Dumitrescu *et al.* 2004, Friesema *et al.* 2004, Schwartz *et al.* 2005), which was formerly clinically described as the Allan–Herndon–Dudley syndrome (AHDS).

MCT8 is a 12 membrane-pass sodium- and ATP-independent transport protein which has been identified earlier as an active T_3 transport protein (Friesema *et al.* 2003). Other members of the MCT gene family, e.g. MCT1 and MCT10, have been shown to transport lactate or amino acids respectively, and MCT10 also transports T_3 (Halestrap & Meredith 2004, Friesema *et al.* 2008). Thyroid hormones have long been considered as hydrophobic molecules able to efficiently cross the plasma membrane, but mounting evidence suggests that T_3 and thyroxine (T_4) need specific transport proteins for membrane crossing (Hennemann *et al.* 2001, Visser *et al.* 2007). Congenital hypothyroidism may lead to severe mental retardation if left untreated. Neonatal screening programs allow us to identify and treat these patients early, and most affected children attain normal intelligence (Grüters *et al.* 2003). In the brain, thyroid hormone is required for the timely migration of neurons, formation of synaptic contacts, and myelination (Zoeller *et al.* 2002).

Since *MCT8* is expressed, among other tissues, in neurons and pituitary cells (Heuer *et al.* 2005), it has been expected that mutations in MCT8 may deprive developing neurons of adequate T_3 signalling leading to mental impairment, albeit, the phenotypes of *MCT8*-deficient patients are not compatible with untreated congenital hypothyroidism (Dumitrescu *et al.* 2004, Friesema *et al.* 2004, Holden *et al.* 2005, Kakinuma *et al.* 2005, Schwartz *et al.* 2005, Maranduba *et al.* 2006, Herzovich *et al.* 2007, Jansen *et al.* 2007). In addition, the apparent lack of feedback inhibition of TSH expression in the pituitary suggests that pituitary thyrotroph cells are not able to correctly sense circulating T_3 levels. In contrast, decreased serum cholesterol and elevated sex hormone binding globulin (SHBG) levels in affected patients point to hepatic hyperthyroidism (Dumitrescu *et al.* 2004, Friesema *et al.* 2004).

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Transgenic mice have been engineered in which the murine *Mct8* gene was disrupted (Dumitrescu *et al.* 2006, Trajkovic *et al.* 2007). These mice exhibit greatly increased serum T_3 in the face of low/normal T_4 and normal TSH levels (Dumitrescu *et al.* 2006, Trajkovic *et al.* 2007). Like the patients, they exhibit correspondingly decreased serum cholesterol. Brain uptake of T_3, but not T_4, is completely inhibited in *Mct8*$^{-/y}$ mice. In line with this observation, expression and activity of Dio2 is increased and Dio3 is decreased. However, surprisingly no neurological phenotype was observed in these mice (Dumitrescu *et al.* 2006, Trajkovic *et al.* 2007), possibly because of neuronal expression of additional T_3 transporters in the mouse (Wirth *et al.* 2009).

Over 20 mutations in *MCT8* have been found in patients so far. Patients are usually identified in their first year of life with severe muscular hypotonia. Most patients never develop speech or independent walking. It was noted that there is some phenotypic variation among patients with different mutations (Schwartz *et al.* 2005, Friesema *et al.* 2006, Frints *et al.* 2008, Jansen *et al.* 2008) – and there is even some variability among the phenotypes of patients within the same family (Frints *et al.* 2008). Phenotypic variation may point to residual T_3 transport activity of mutated *MCT8* depending on the type of mutation. This theoretically opens the possibility to aim therapeutically at an increase of the activity of mutated *MCT8*. Potential treatment strategies for patients with large genomic deletions or frameshift mutations would require gene therapy in the nervous system and are therefore not established. In contrast, since it has been suggested that some *MCT8* mutations prevent the protein from reaching the plasma membrane (Jansen *et al.* 2008), the application of chemical or pharmacological chaperones may increase plasma membrane trafficking of mutated *MCT8* (Bernier *et al.* 2004). In order to study the molecular defects of individual mutant MCT8 proteins and their response towards pharmacological treatments *in vitro*, we have established stable cell lines expressing a series of 12 missense *MCT8* mutations found in AHDS patients and analyzed their protein expression, T_3-uptake activity, and cell surface exposure. We found that most of the missense mutants were readily expressed in several cell types. We then observed that a number of these mutants are also able to transport T_3, but that this activity was related to their surface expression, which – as we found – depends considerably on the cell type studied. Kinetic analyses of partially active *MCT8* mutants showed that some mutations primarily affect membrane exposure (e.g. L568P), while others more directly impinge on T_3 transport (e.g. ins235V). These findings may bear significance for future treatment strategies, since the results allow distinguish missense mutations in *MCT8* according to their molecular pathomechanisms.

Materials and methods

Cloning, site-directed mutagenesis, and transient expression

Mutations were introduced into human (amino acids 1–613) N-terminally HA-tagged *MCT8* cDNA by overlap extension PCR using the primers hMCT8-HindIII-HA-fwd: 5′-AAGCTTGAC***ATG***TACCCATACGACG-TCCCAGACTACGCTATGGGGAGAGGAGGAG-3′ and genrev 5′-TTAGATTGGTTCC**TCA**GGGTTGG-3′ with primers introducing the mutations as listed in Supplementary Table 1, see supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol43/issue6/. In the primer sequences, the start and stop codons are printed in bold face and the HA-tag is denoted in italics. The PCR fragments were subcloned into pGEM-Teasy (Promega), sequenced from both sides, and then cloned into pcDNA3.1 (Invitrogen) using the restriction enzymes HindIII and XbaI. Expression of full-length *MCT8* was verified by transient transfection into cultured cells followed by immunoblotting with an antiserum made in rabbit and directed against a recombinant N-terminal peptide of human MCT8 (amino acids 52–155; ATLAS, Stockholm, Sweden) or the HA-tag (not shown). Stability of variant MCT8 protein was tested by incubating freshly harvested membrane fraction protein lysates at 37 °C for up to 3 h with and without the addition of a protease inhibitor cocktail (Complete Mini, Roche) followed by immunoblotting (Supplementary Figure 1, see supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol43/issue6/).

Cell culture, transfection, and generation of stable cell lines

Cell lines were cultured in a humidified atmosphere at 37 °C and 5% CO_2. The media used were DMEM-F12 (1:1), 5% FCS and Pen/Strep (only JEG1 and MDCK1) for HEK-293, HeLa, JEG1 and MDCK1 cells respectively. JEG1 and MDCK1 cells were selected for low background T_3 uptake activity (not shown) and lack of endogenous MCT8 expression as judged by western and northern blotting (Fig. 1 and Supplementary Figure 2, see supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol43/issue6/). Cells at 50% confluency were transfected with expression plasmids encoding wild-type (WT), mutants, and empty vector and exposed to selective pressure with 200 μg G418/ml. After 2 weeks, colonies derived from single clones were picked and subcultured. Three to six clones of each variant in both

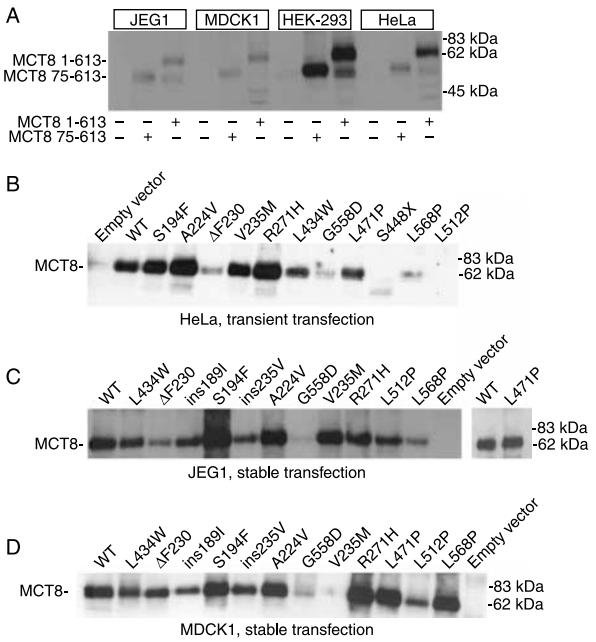

Figure 1 Expression of transiently transfected MCT8 variants in mammalian cell lines. (A) Cell lines were transfected with human MCT8 (1–613) and MCT8 (75–613) cDNA expression vectors and expression of MCT8 protein was determined by immunoblotting. Initiation at Met75 occurs also in cells transfected with full length (1–613) MCT8 depending on cell type. '+' and '−' indicate the MCT8 construct transfected. Molecular mass markers are indicated on the right. (B) Transient transfection into HeLa cells of MCT8 (amino acids 1–613) variants found in human patients. All missense mutants except L512P were readily detectable as full length MCT8 protein. WT, wild-type MCT8. (C) Stable transfection into JEG1 cells of MCT8 (amino acids 1–613) variants found in human patients. All missense mutants including L512P were readily detectable as full length MCT8 protein. (D) Stable transfection into canine MDCK1 cells of MCT8 (amino acids 1–613) variants found in human patients. All missense mutants except L512P were readily detectable as full length MCT8 protein.

cell types were expanded in six well plates, protein harvested, dot blots performed all in parallel for each cell type and probed with an anti-MCT8 antibody. Three clones each expressing levels of MCT8 similar to WT were selected and stored in liquid nitrogen. One clone for each variant was randomly chosen from the three frozen, G418 withdrawn, continued MCT8 expression ascertained by SDS-PAGE and immunoblotting, and used for the following experiments.

Northern blotting

For northern blot analysis, total RNA was isolated from stably transfected JEG1 and MDCK1 cells using peqGOLD TriFast (PeqLab, Erlangen, Germany). For electrophoresis 15 μg of total RNA were separated using a 2% agarose gel with 4·3% formaldehyde followed by blotting onto a nylon membrane (Schleicher & Schuell, Dassel, Germany). ^{32}P-labeled RNA probe was generated by labeling a 1·3 kb BamHI/XbaI cDNA probe cut from hMCT8-pcDNA3.1 according to the manufacturer's protocol (NEBlot Kit; New England Biolabs, Frankfurt, Germany).

T_3 uptake assay and chaperone use

One day before the experiments, cells were trypsinized, counted, 100 000 cells were set aside for western blotting against MCT8, and 200 000 cells per well were seeded into 12-well plates. ^{125}I–T_3 (GE Healthcare, Munich, Germany; 11·8 MBq/ml, 150 μCi/μg) was purified from iodide by reverse phase chromatography and eluted in EtOH:NH_3 (49:1) which was removed by a gentle stream of nitrogen gas. On the day of the experiment, the cell culture medium was replaced by DMEM F12 (1:1) without serum. The cells were exposed to 10 nM of ^{125}I–T_3 in DMEM:F12 for 3 (MDCK1 cells) or 15 min (JEG1 cells as determined in pilot studies), and the medium removed. After a single wash with PBS, the cells were lysed in 40 mM NaOH, and cell associated radioactivity was quantified with a gamma counter (Wizard; Perkin Elmer, Rodgau, Germany). Each experiment was performed on at least three different days with different batches of cells in triplicate. In order to pool the data, radioactivity associated with empty vector-transfected clones was subtracted as background and the activity associated with the WT MCT8 clone was defined as 100%. Specific T_3-uptake activity was calculated by dividing the % uptake by relative protein expression on the plasma membrane (mean of two to three experiments, see below).

For determination of apparent K_M values of WT and mutant MCT8, MCDK1 cells stably expressing WT, ins235V, R271H, and L568P were selected and incubated for 3 min with ^{125}I–T_3 at concentrations ranging from 500 nM to 12 μM. A Michaelis-Menten mechanism was assumed for calculations (Graph Pad 4.0; Graph Pad, La Jolla, CA, USA).

In order to test the effect of chemical chaperones, the following substances were added to the cells 24 h before performing uptake assay: 80 mM trimethylamine-*N*-oxide (TMAO, Sigma), 50 mM glycerol (Sigma), 2 mM tauryl-ursodeoxycholic acid (TUDCA, Sigma) or 2 mM 4-phenyl-butyric acid (4-PBA, Tocris, BIOZOL, Eching, Germany). In order to test whether T_3 can serve as a pharmacological chaperone, cells were pre-incubated with 10 nM T_3 for 30 min before medium exchange and subsequent T_3 uptake measurements.

Surface biotinylation and western blotting

Surface biotinylation was performed on cells grown in 75 cm^2 flasks using the Pierce Cell Surface Protein Isolation Kit according to the manufacturer's

instructions with minor modifications as follows. We found that half the amount of cells recommended are still sufficient to detect biotinylated MCT8 and thus used only half volumes of reagents where appropriate. After elution from the avidin resin with dithiothreitol (DTT), we precipitated the biotinylated protein with acetone, removed excess DTT by washing (in order to reduce interference with the Bradford protein assay) and determined the protein content of the eluate. Equal amounts of 5 μg of biotinylated protein (or 80 μg of total cellular protein fraction) were then applied to 10% SDS-PAGE gels, separated, electroblotted, and probed with the MCT8 antibody. Relative surface translocation was assessed by densitometry of X-ray films (ImageJ; NIH, Bethesda, MD, USA) and expressed relative to WT MCT8. Only full length MCT8 was considered, excluding degradation products. Equal protein loading on all lanes of the membranes was ascertained by reversible Amido black B staining. Every experiment in each cell type was performed at least twice with similar results. Equal loading of lanes was further tested by probing with β-actin antibody.

Results

Efficient expression of MCT8 variants in several cell lines

The open reading frames of primate *MCT8* genes are longer than in most other mammalian species so far analyzed. Met75 in human *MCT8* is identical with Met1 in rodent, cat, and dog *MCT8*. Therefore, there is uncertainty regarding the human translational start codon *in vivo*. We thus transiently transfected long (amino acids 1–613) and short (amino acids 75–613) variants of WT human *MCT8* cDNA into four different human and canine cell lines. Interestingly, all cell types were able to initiate translation at Met75 from the vector encoding (1–613) *MCT8*, albeit at lower rates (Fig. 1A). We then introduced into full-length (1–613) human *MCT8* cDNA a series of 12 missense mutations reflecting the base changes found in patients afflicted with AHDS (OMIM 300523). In contrast to earlier reports (Jansen *et al.* 2005, 2008), most of the missense mutants were readily detectable by western blotting when transiently expressed in HeLa, JEG1, and MDCK1 cell lines (Fig. 1B and not shown). We then speculated that the stability of the MCT8 mutant proteins may be shortened and therefore we incubated membrane protein fractions from HeLa cells transiently transfected with MCT8 expression constructs at 37 °C for up to 3 h in the presence or absence of protease inhibitors. Most mutants demonstrated almost identical stability as WT MCT8, while L471P and ΔF230 appeared to be slightly less stable *in vitro* (Supplementary Figure 1).

Cell surface translocation and T_3-uptake activity of MCT8 variants

We then established stable cell lines for all *MCT8* variants in human chorioncarcinoma JEG1 cells and canine kidney MDCK1 cells (Fig. 1C and D; Supplementary Figure 2). Using these cell lines, we compared ^{125}I–T_3 uptake mediated by mutant MCT8 with WT MCT8. In order to express T_3-uptake as specific activity relative to MCT8 at the plasma membrane, we performed selective biotinylation of cell surface-exposed proteins and quantified the amount of biotinylated variant MCT8 in relation to WT MCT8. We noticed that specific T_3-uptake by mutant MCT8 was different between JEG1 (Fig. 2A) and MDCK1 cells (Fig. 2B). Such differences may rest in part with differential protein expression or stability. For example, the G558D mutant is apparently unstable in JEG1, but not in MDCK and HeLa cells (see also Fig. 1 and Supplementary Figure 1). In addition, while in JEG1 cells only the R271H mutant was active in both cell lines similarly as previously reported (Jansen *et al.* 2007). In MDCK1 cells, ins235V and L568P, in addition to R271H, were highly active. The difference is likely associated with plasma membrane translocation and protein degradation. For example, L568P efficiently translocates to the plasma membrane in MDCK1, but not JEG1 cells. Similarly, ins235V translocates only moderately to the membrane in JEG1, and is partially degraded. Thus, the disease mechanism for some mutant MCT8 proteins involves cell type-specific deficiency of plasma membrane translocation which secondarily impinges on transport activity.

There are MCT8 variants which are primarily defective in T_3 transport, even if they are efficiently translocated to the plasma membrane. For example, membrane expression of ins189I, S194F, A224V, ΔF230, and L512P is apparently possible in both cell lines, although the respective mutants are essentially inactive in T_3-uptake.

Biochemical properties of MCT8 variants

In order to better characterize the biochemical properties of MCT8 mutants which retained T_3 transport activity, we undertook a kinetic study. The K_M value for T_3 in uptake assays is an intrinsic feature of the transporter molecule and is independent of the number of molecules exposed to the cell surface. The V_{max}, in contrast, depends on the number of molecules involved. In order to adjust for slight quantitative differences in plasma membrane expression, we normalized T_3 uptake activity to relative amounts of MCT8 at the plasma membrane as above and determined V_{max} from the specific uptake activities in MDCK1 cells (Fig. 3). Both ins235V ($K_M^{ins235V}$ 3·2 μM) and R271H (K_M^{R271H} 1·9 μM) mutants exhibited

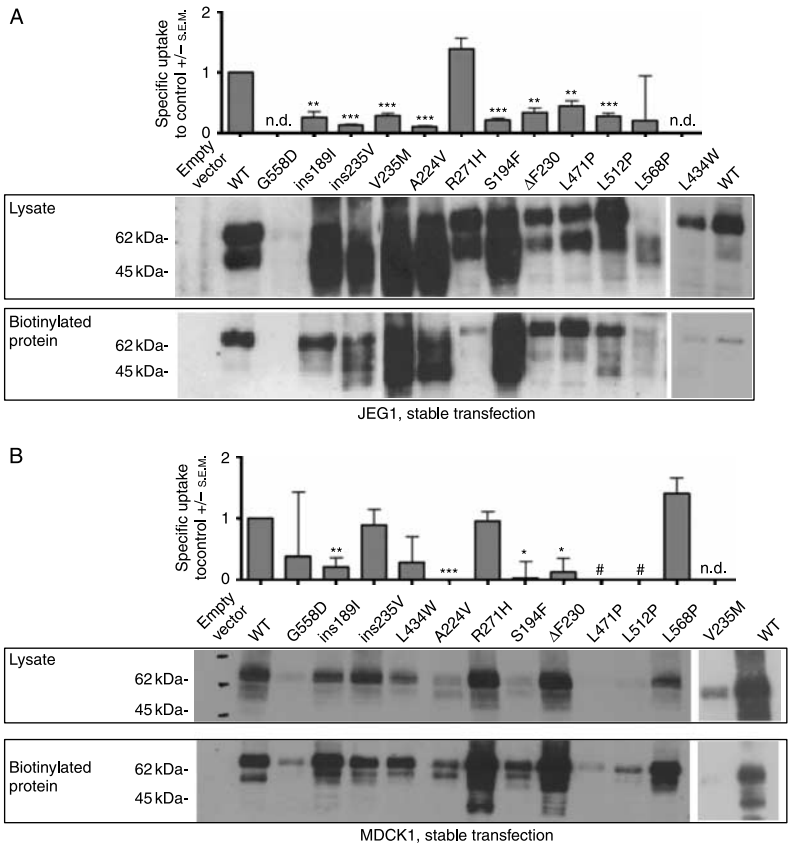

Figure 2 MCT8 surface translocation and ^{125}I–T_3 uptake depends on the cell line. JEG1 (A) and MDCK1 (B) cells were stably transfected with expression vectors encoding MCT8 variants identified in human patients. Whole cellular lysates (top panels) were compared by immunoblotting for MCT8 with the biotinylated and affinity-purified plasma membrane protein fraction (bottom panels). Uptake of cells transfected with empty vector was considered background and subtracted. Wild-type (WT) MCT8 T_3 uptake was set to 100% and the data were normalized relative to MCT8 plasma membrane exposure to yield a measure for the specific activity. Data represent mean ± S.E.M. of at least three independent T_3-uptake experiments performed in triplicate and two biotinylation studies per cell type. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$ different from WT. n.d., not determined (because of absent plasma membrane expression (G558D, V235M) or erratic replicates (L434W)). #Values negative after background subtraction, but reproducible).

increased K_M values for T_3, indicating that these mutations directly impinge on the transport mechanism possibly by distorting the native three-dimensional structure. The K_M of the L568P mutant was not different from the K_M of WT MCT8 in this test system (K_M^{WT} 0·86 μM versus K_M^{L568P} 0·87 μM), but its V_{max} was greatly decreased (Fig. 3B).

Effects of pharmacological chaperones on T_3 transport activity of mutant MCT8

If the stability of the three-dimensional structure of a membrane protein is significantly reduced, it may be retained in the endoplasmic reticulum (ER) by quality control mechanisms or even be degraded. We therefore tested whether chemical chaperones previously shown to enhance surface expression of mutant membrane proteins, are able to increase T_3 transport by MCT8 mutants in MDCK1 cells. For this experiment, we selected mutants that retained partial activity and showed surface expression in at least one cell type, and incubated the cells with chemical chaperones for 24 h before the T_3 uptake assay. Neither chemical chaperone, 4-PBA, TUDCA, glycerol, nor TMAO did enhance cellular T_3 uptake in the selected mutant MCT8 cell lines (Fig. 4A). Some mutant integral membrane proteins adopt a native conformation in the presence of a ligand and thus pass quality control in the ER. Since we knew that MDCK1 cells exhibit low T_3 uptake activity in the absence of transfected MCT8, we

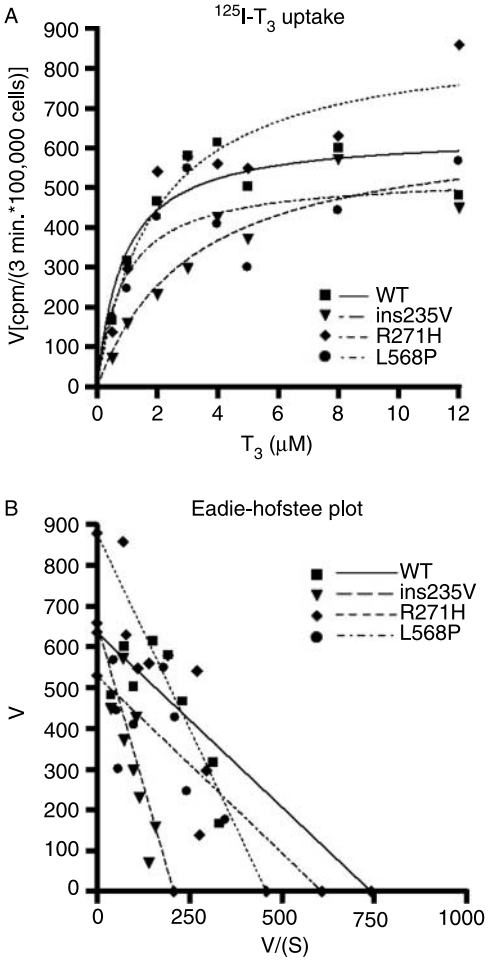

Figure 3 Kinetic analysis of ^{125}I–T$_3$ uptake by mutant MCT8 proteins in MDCK1 cells. (A) Cell-associated radioactivity depending on substrate (T$_3$) concentration. Lines represent fitted binding isotherms. All data points were determined in triplicate and the mean values are plotted. WT (wild-type MCT); ins235V; R271H; L568P; ΔF230 and S194F were also tested, but did not show T$_3$ uptake above background. The experiment was performed two times with similar results. (B) Eadie–Hofstee plot based on data from (A). L568P displays the same K_M value as WT. While R271H displays an intermediate K_M value, ins235V exhibits the highest K_M value. K_M values were not approximated from the plot, but were directly calculated from the data using GraphPad software.

speculated that T$_3$ may be available to unstable MCT8 mutants retained in the ER. After an initial screening comprising all mutant MCT8 cell lines, we selected G558D and L471P and pre-incubated MDCK1 cells transgenic for these mutants with 10 nM T$_3$ before performing T$_3$ uptake measurements. While no effect was observed for L471P, we observed a small, but signficant and reproducible, increase in T$_3$ uptake for G558D with T$_3$ pre-treatment (Fig. 4B). However, even with T$_3$ pre-incubation, we were not able to determine a meaningful K_M value for the G558D mutant.

Discussion

Mutations in the *MCT8* gene in humans lead to a severe form of mental and psychomotor retardation. To date, no efficient treatment is available. The observation that patients carrying the S194F, L434W, and L568P mutations may develop rudimentary speech or independent, although awkward, walking (Schwartz *et al.* 2005) suggests that at least these MCT8 variants retain some residual transport activity *in vivo*. Consistent with these clinical observations, these mutants retained residual transport activity in JEG3 cells (Jansen *et al.* 2008). We show here that MCT8 mutants ins235V, R271H and L568P exhibit significant T$_3$ transport activity in JEG1 or MDCK1 cells. We also show that differential activity of the mutants in the two cell lines, depends on surface translocation to the plasma membrane. In contrast to results obtained in JEG3 cells (Jansen *et al.* 2008), we were not able to detect significant residual activity of S194F and L434W, although at least S194F was efficiently transported to the plasma membrane in both cell lines. This is reminiscent of the report of Jansen *et al.* in which affinity to bromoacetyl-T$_3$ was high for L568P, but low for the S194F und L434W mutants.

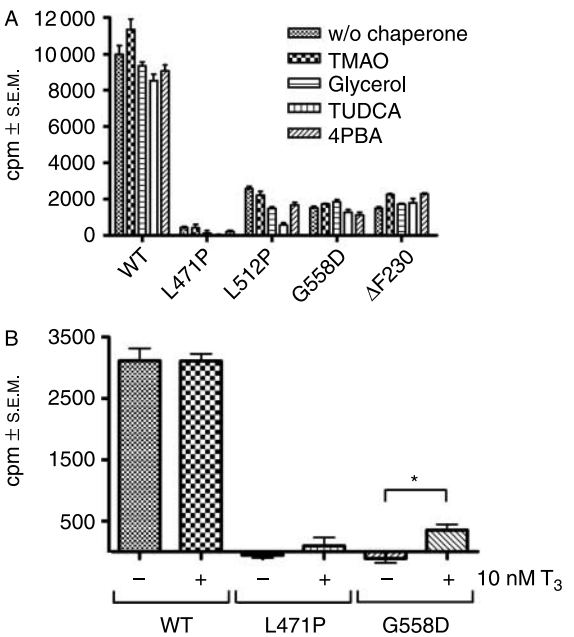

Figure 4 Effect of pharmacological treatments on T$_3$ uptake mediated by mutant MCT8. (A) Twenty-four hours preincubation with chemical chaperones trimethylaminoxide (TMAO), glycerol, tauroursodeoxycholic acid (TUDCA), or 4-phenylbutyric acid (4-PBA) was not effective in enhancing T$_3$ uptake mediated by mutant MCT8. (B) Thirty minutes preincubation with T$_3$ as pharmacological chaperone encreased T$_3$ uptake by G558D, but not L471P mutant MCT8.

The mutants ins235V, R271H, and L568P exhibit normal or close to normal K_M values and thus we demonstrated for the first time that MCT8 mutants so far considered inactive may act as functional T_3 transporters, if their plasma membrane translocation is successful (Fig. 5). Plasma membrane translocation of multipass membrane proteins is often impaired in genetic diseases affecting integral membrane proteins, e.g. in cystic fibrosis or diabetes insipidus. Therapies are being developed to increase the fraction of (mutant) protein reaching the plasma membrane *in vivo* (Burrows *et al.* 2000). Folding of multipass transmembrane proteins is a complicated and still incompletely understood process (von Heijne 2006). Unstable or unsucessfully assembled membrane proteins are identified in the ER and are degraded by cytosolic proteasomes after a process called retro-translocation – subsequently called the endoplasmic reticulum associated degradation (ERAD) pathway (Kleizen & Braakman 2004). In an attempt to test for possible induction of the unfolded-protein-response by mutant MCT8 proteins, we tested in all stable MDCK1 cell lines for the expression of *XBP1s* and *Chop* transcripts by RT-PCR. We found in none of the mutant cell lines increased expression of these transcripts (data not shown) in accordance with another report (James *et al.* 2008).

Reduction of the dielectric constant of the cellular interior caused by so-called 'chemical chaperones' (glycerol, TMAO, TUDCA, 4-PBA) diminishes the thermodynamic penalty for non-perfect protein conformations and allows mutant proteins to evade the ERAD machinery (Schulz *et al.* 2002, Bernier *et al.* 2004). Another potential strategy to increase the expression of mutated plasma membrane proteins is to present a membrane permeant ligand for the target protein, a 'pharmacological chaperone' that induces its correct three-dimensional folding (Bernier *et al.* 2004). Such ligands may even represent enzymatic inhibitors, but their tight binding intracellularly provides the extra free energy for stabilization of the target protein. Providing T_3 preincubation to mutant MCT8 was the only manipulation effective in significantly enhancing T_3 uptake, although only in one mutant, G558D. These findings are disappointing with regard to the lack of therapy for patients suffering from mutations in *MCT8*.

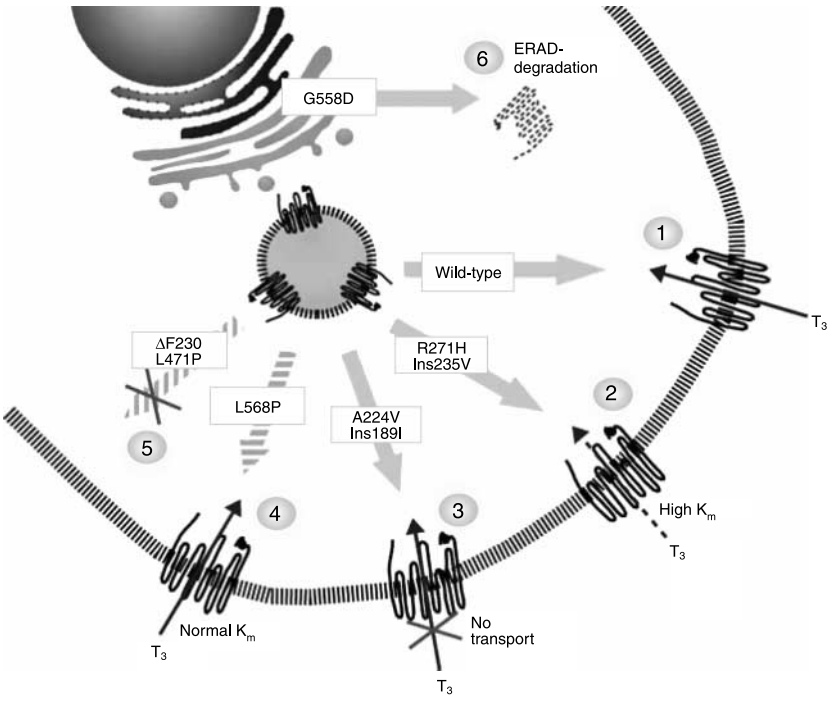

Figure 5 Summary of biochemical phenotypes of mutant MCT8. (1) Wild-type MCT8 mediates cellular T_3 uptake at the plasma membrane. (2) Some MCT8 mutants display increased K_M for T_3 uptake, but are partially functional (e.g. R271H). (3) Other mutants may translocate to the plasma membrane, but are entirely deficient in T_3 transport (A224V). (4) Mutants like L568P are enzymatically fully active, but depending on the cellular environment, may not translocate to the plasma membrane. (5) Some mutants are incapable of plasma membrane translocation. (6) The G558D mutant is expressed at low levels or subject to degradation, but a small fraction of molecules may reach the plasma membrane and is active as T_3 transporter. Incubation with T_3 as pharmacological chaperone enhances T_3 uptake by G558D (based on results from MDCK1 cells).

While the search for more effective drugs that may enhance T_3 transport by mutant MCT8 should continue, it is equally important to identify those patients who likely do not benefit and who may suffer potential side effects of the therapy without the chance of therapeutic success. According to our data, carriers of e.g. ins189I and A224V mutations are not likely to benefit from any therapy aimed at increasing plasma membrane expression of MCT8, since their MCT8 protein is not deficient in surface translocation and yet is entirely inactive.

Other experimental therapies for *MCT8*-deficient patients included the attempt to override the apparent resistance to thyroid hormone by further increasing T_3 in the patient, but no clinical improvement could be demonstrated (Biebermann *et al.* 2005). In contrast, Wemeau *et al.* ameliorated the thyrotoxic state by combined T_4/propyl-thio-uracil (PTU) treatment, because they wanted to achieve weight gain in the cachexic patient with the R271H mutation. Although their treatment did not improve the neurological impairment of the patient, significant weight gain was achieved (Wemeau *et al.* 2008).

One alternative possible treatment option has not been explored in patients to our knowledge. Synthetic ligands for thyroid hormone receptors may be able to cross the blood–brain barrier, and possibly the neuronal plasma membrane, independent of MCT8. Since it has been shown that under conditions of hypothyroidism or due to thyroid hormone receptor mutations unliganded nuclear thyroid hormone receptors exert repressive action on gene expression (Morte *et al.* 2002), providing to *MCT8*-deficient patients a synthetic ligand able to reach neuronal nuclei without the aid of MCT8, which may at least alleviate that part of the neurological deficits not directly caused by developmental derangements. Application of such compounds, together with T_4/PTU, may restore peripheral euthyroidism and still increase thyroid hormone receptor activity in the brain.

In the report of Frints *et al.* (2008) a family was described carrying a missense mutation in the first ATG codon of *MCT8*. While one sibling exhibited a phenotype compatible with AHDS, a brother carrying the same mutation was apparently not affected. It is still not clear whether the inactivation of the first ATG in *MCT8* of the patient was the sole reason for his mental retardation, but two observations should be considered: primates may initiate MCT8 translation at the 'first' ATG, while rodents, for example, utilize the ATG homologous to Met75 in humans. Initial experiments on T_3 transport by Friesema *et al.* (2003) and Biebermann *et al.* (2005) used the rat and the (75–613) version of human *MCT8* demonstrating significant T_3-transport activity of the shorter MCT8 isoform. Thus, we speculate that the healthy sibling, unlike his deceased brother, may be able to initiate MCT8 translation at Met75. In support of this, we showed that, *in vitro*, several human cell lines produced a significant fraction of (75–613) MCT8 protein along with full length (1–613) MCT8 (compare Figs 1A and D and 2).

Considering the cell type differences in MCT8 translocation to the plasma membrane, demonstrated here for the first time, may help explain why the levels of plasma T_3 do not correlate with mental development in *MCT8*-deficient patients (Friesema *et al.* 2006). The cellular make-up of protein-folding chaperones or ERAD components likely differs between neurons, pituitary thyrotrophs, and hepatocytes and thus different MCT8 missense mutants may be handled differently in the various cells. For example, if mutant MCT8 partially works in some neurons, but not in the pituitary, T_3 will be very high, but mental impairment less severe. In the reverse situation, plasma T_3 will be closer to normal, but the mental impairment maximal as in null mutations. Such differences may, however, complicate each attempt to design a pharmacological treatment strategy for *MCT8*-deficient patients and should be considered in experimental design and the choice of analytical parameters.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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