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Effective Cellular Uptake and Efflux of Thyroid Hormone by Human Monocarboxylate Transporter 10

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Cellular entry of thyroid hormone is mediated by plasma membrane transporters, among others a T-type (aromatic) amino acid transporter. Monocarboxylate transporter 10 (MCT10) has been reported to transport aromatic amino acids but not iodothyronines. Within the MCT family, MCT10 is most homologous to MCT8, which is a very important iodothyronine transporter but does not transport amino acids. In view of this paradox, we decided to reinvestigate the possible transport of thyroid hormone by human (h) MCT10 in comparison with hMCT8. Transfection of COS1 cells with hMCT10 cDNA resulted in 1) the production of an approximately 55 kDa protein located to the plasma membrane as shown by immunoblotting and confocal microscopy, 2) a strong increase in the affinity labeling of intracellular type I deiodinase by *N*-bromoacetyl-[^{125}I]T_3, 3) a marked stimulation of cellular T_4 and, particularly, T_3 uptake, 4) a significant inhibition of T_3 uptake by phenylalanine, tyrosine, and tryptophan of 12.5%, 22.2%, and 51.4%, respectively, and 5) a marked increase in the intracellular deiodination of T_4 and T_3 by different deiodinases. Cotransfection studies using the cytosolic thyroid hormone-binding protein μ-crystallin (CRYM) indicated that hMCT10 facilitates both cellular uptake and efflux of T_4 and T_3. In the absence of CRYM, hMCT10 and hMCT8 increased T_3 uptake after 5 min incubation up to 4.0- and 1.9-fold, and in the presence of CRYM up to 6.9- and 5.8-fold, respectively. hMCT10 was less active toward T_4 than hMCT8. These findings establish that hMCT10 is at least as active a thyroid hormone transporter as hMCT8, and that both transporters facilitate iodothyronine uptake as well as efflux. (*Molecular Endocrinology* 22: 1357–1369, 2008)

THYROID HORMONE IS metabolized and exerts its actions intracellularly, processes that require the transport of extracellular iodothyronines across the plasma membrane (1, 2). Diffusion of iodothyronines across the lipid bilayer of cell membranes is limited, if not impossible, and transmembrane transport of thyroid hormone is predominantly facilitated by transporters (3). Different types of transporters are likely to be involved in the uptake of thyroid hormone in different tissues (3). A number of these have recently been characterized at the molecular level, including the Na/taurocholate-cotransporting polypeptide, different members of the (Na-independent) organic anion transporting polypeptide family, the heterodimeric L-type amino acid transporter, and fatty acid translocase (4, 5).

Work in particular by the group of Francon and Blondeau (6, 7), Kemp and Taylor (8), Powell *et al.* (9), and Ritchie *et al*. (10) has strongly suggested the involvement of a T-type amino acid transporter in the uptake of T_4 and T_3 in different tissues. A T-type amino acid transporter (TAT) facilitates transport of aromatic amino acids, and specific interaction between cellular uptake of T_3 and Trp has been documented in red blood cells and other tissues (6–10). Recently, one such TAT, termed "TAT1," has been characterized in rats and humans (11, 12). Although TAT1 indeed facilitates influx and efflux of Phe, Tyr, Trp, and Dopa, it was reported to be inactive toward iodothyronines (11–13). TAT1 is also known as MCT10 (SLC16A10) because it is a member of the monocarboxylate transporter (MCT) family, so called because the first four members of this family transport lactate, pyruvate, and other monocarboxylates in a H^+-dependent manner (14).

Among other members of the MCT family, MCT8 shows by far the highest homology with MCT10, which prompted us to test the possibility that MCT8 is the long-sought T-type amino acid transporter that also facilitates cellular uptake of iodothyronines. Indeed both rat (r) and human (h) MCT8 have been shown to be active and specific iodothyronine transporters, although they do not transport (aromatic) amino acids (15, 16). The pathophysiological relevance of MCT8 has been demonstrated subsequently in male patients with a syndrome, also known as the Allan-Herndon-

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Abbreviations: CRYM, μ-Crystallin; DAPI, 4′,6-diamidino-2-phenylindole; D-PBS, Dulbecco's PBS; HEK, human embryonic kidney; MCT, monocarboxylate transporter; siRNA, short interfering RNA; TAT, T-type amino acid transporter; TMD, transmembrane domain.

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Dudley syndrome (17) combining severe psychomotor retardation and elevated serum T_3 concentrations (18–27). The gene coding for MCT8 is located on the X chromosome, and in all patients with this form of X-linked psychomotor retardation mutations in *MCT8* have been identified (18–27).

The *hMCT10* gene is located on chromosome 6, and the structure of this gene is very similar to that of the *hMCT8* gene (12, 28). Both genes consist of six exons and five introns, with a particularly large (~100 kb) first intron. The major form of the hMCT8 protein consists of 539 amino acids and the hMCT10 protein of 515 amino acids; both proteins contain 12 putative transmembrane domains (TMDs). Both the N- and C-terminal domains are predicted to be located on the inside of the plasma membrane (4, 29). The N-terminal regions of both proteins harbor a PEST domain rich in Pro (P), Glu (E), Ser (S), and Thr (T) residues, which may play a role in the regulation of the turnover of these proteins (30).

The lack of iodothyronine transport reported for MCT10 is surprising in view of the documented involvement of a T-type amino acid transporter in tissue uptake of thyroid hormone as well as the homology between MCT10 and MCT8. Therefore, we decided to reinvestigate possible iodothyronine transport by hMCT10 in comparison with hMCT8. These studies were carried out in mammalian cells transfected with cDNA coding for hMCT8 or hMCT10. To study the effects of an increase in the intracellular thyroid hormone-binding capacity, cells were cotransfected with cDNA coding for the cytosolic thyroid hormone-binding protein μ-crystallin (CRYM) (31–33). Our findings demonstrate that both hMCT8 and hMCT10 facilitate bidirectional transport of T_4 and, in particular, T_3 across the plasma membrane, and that hMCT10 transports T_3 even better than hMCT8.

RESULTS

Among the 14 members of the MCT family, hMCT10 is most homologous with hMCT8; the amino acid identity of hMCT10 with hMCT8 amounts to 49%, whereas with the other hMCTs it varies between 21% (hMCT12) and 34% (hMCT3). Figure 1 shows the amino acid sequence alignment of hMCT8 and hMCT10. In this figure are also indicated the putative TMDs, reflecting our interpretation of the predictions by various TMD prediction programs which showed considerable variation in the number (11 or 12) and location of the putative TMDs in both proteins. Obviously, the highest degree of amino acid identity between hMCT8 and hMCT10 exists in the putative TMDs and intervening loops and much less so in the N- and C-terminal domains. Considering only this core domain, the amino acid identity between hMCT8 and hMCT10 amounts to 58%.

Figure 2 shows a phylogenetic tree of a selection of proteins encoded by genes identified in GenBank because of their homology with the core hMCT8 and

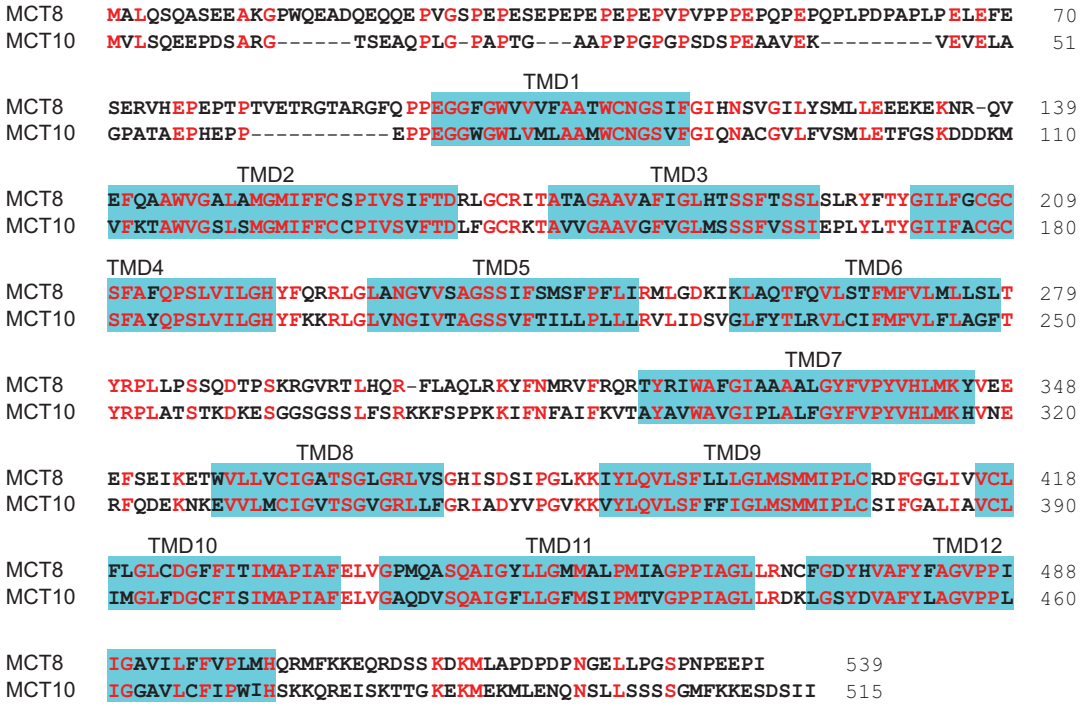

Fig. 1. Alignment of the Amino Acid Sequences of hMCT8 and hMCT10

Identical amino acids occupying corresponding positions in these proteins are indicated in *red*. The putative 12 TMDs are indicated by *blue* shading.

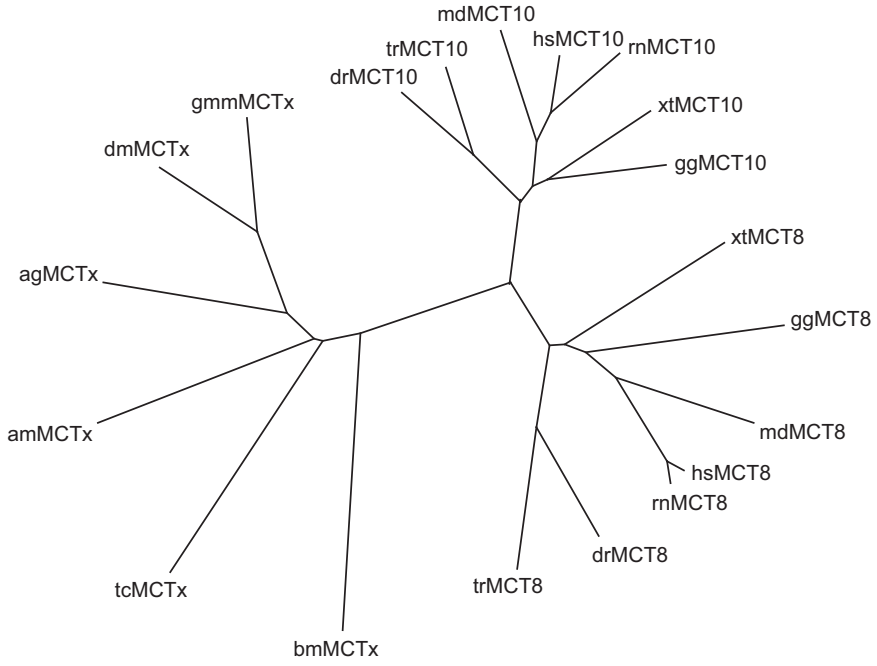

Fig. 2. Phylogenetic Tree of the MCT8/MCT10 Protein Family

The construction of the tree is based on amino acid sequences from the following species: hs, *Homo sapiens* (human); rn, *Rattus norvegicus* (rat); md, *Monodelphis domestica* (opossum); gg, *Gallus gallus* (chicken); xt, *Xenopus tropicalis* (frog); dr, *Danio rerio* (zebrafish); tr, *Takifugu rubripes* (pufferfish); gmm, *Glossina morsitans morsitans* (tsetse fly); dm, *Drosophila melanogaster* (fruitfly); ag, *Anopheles gambiae* (mosquito); am, *Apis mellifera* (honey bee); tc, *Tribolium castaneum* (beetle); bm, *Bombyx mori* (silkworm)

hMCT10 sequences using the tBLASTn program. This partial tree includes two mammals (human, rat), a marsupial (opossum), an amphibian (*Xenopus tropicalis*), a bird (chicken), and two teleost fish (zebrafish and fugu). The amino acid identity between the core sequences varies from 63–96% within the MCT8 subfamily, from 70–87% within the MCT10 subfamily, and from 50–60% between these subfamilies.

Surprisingly, several genes were identified in insects (flies, mosquitoes, bee, beetle, and silkworm) that showed high amino acid identities among each other (40–81%) and with members of the MCT8 (34–41%) and the MCT10 (40–46%) subfamilies. Homologous genes were also identified in two prevertebrate marine organisms, the sea squirt (*Ciona intestinalis*) and the sea urchin (*Strongylocentratus purpurata*), with core amino acid identities of 29–32% and 38–44%, respectively, with MCT8 and MCT10 proteins.

The amino acid sequences of hMCT8 and hMCT10 do not contain consensus N-glycosylation sites. Previous studies with cells transfected with hMCT8 cDNA have indicated the production of MCT8 protein with a molecular mass of 61 kDa, the expected size for a nonglycosylated protein of 539 amino acids. This is again illustrated in Fig. 3, which also demonstrates that transfection of COS1 cells with pcDNA3.1.hMCT10 results in the expression of a protein with a molecular mass of 55 kDa, corresponding to the size of a 515-amino acid protein. Transfection efficiency was determined using a yellow fluorescent protein-coupled hMCT8 construct and found to be maximally 25% (data not shown). Little or no endogenous monkey MCT10 or MCT8 protein could be detected in nontransfected COS1 cells, although relatively high endogenous amounts of MCT10 and MCT8 mRNA were expressed (data not shown and Ref. 16). The two antibodies also show no cross-reactivity because no hMCT8 protein is detected with antibody 1758 against hMCT10 and no hMCT10 protein is detected with antibody 1306 against hMCT8.

Immunocytochemistry of COS1 cells transiently overexpressing hMCT10 shows the cellular localization of MCT10 at the plasma membrane (Fig. 4, *left panel*) as is also shown for the plasma membrane marker ZO-1 (Fig. 4, *middle panel*). The *right panel* of Fig. 4 shows the merged images of hMCT10 and ZO-1 together with the nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). Similar conclusions were drawn from experiments using JEG3 cells (data not shown).

Native and recombinant hMCT8 has been identified by incubation of intact nontransfected and hMCT8-transfected cells with the affinity label BrAc[^{125}I]T_3 (16). This is also a highly effective and specific affinity label for the type 1 iodothyronine deiodinase (D1) (34). Therefore, we tested the possible affinity labeling of exogenous hMCT10 in COS1 cells transfected with hMCT10 cDNA. The results indicate that, in contrast to

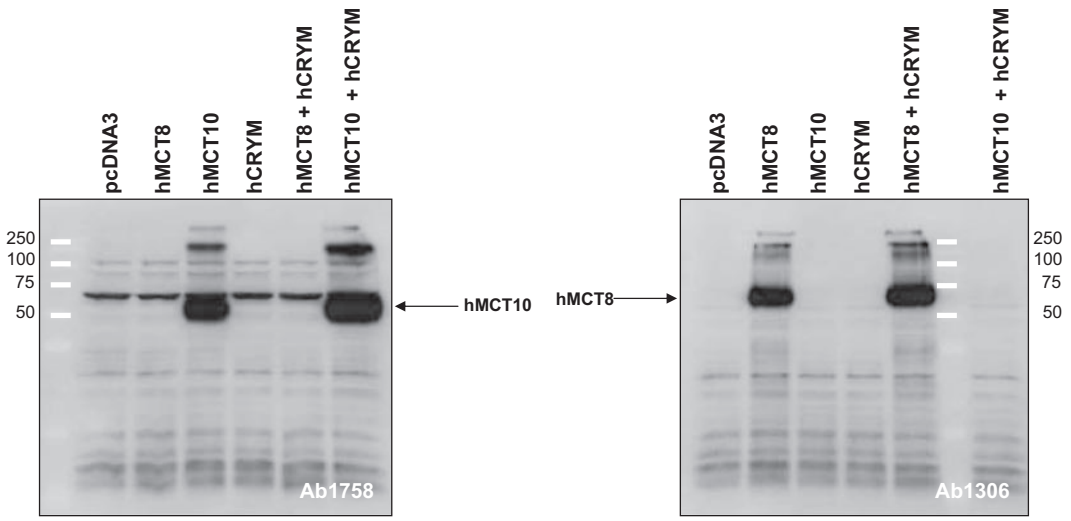

Fig. 3. Immunoblot of hMCT8 and hMCT10 Protein in Transfected COS1 Cells

Left, Staining with polyclonal anti-hMCT10 antibody 1758. Specific bands of approximately 55 and 240 kDa are detected in hMCT10 but not in hMCT8 transfected cells, irrespective of cotransfection with hCRYM. *Right*, Staining with polyclonal anti-hMCT8 antibody 1306. Specific bands of approximately 60 and 240 kDa are detected in hMCT8 but not in hMCT10 transfected cells, irrespective of cotransfection with hCRYM. Ab, Antibody.

hMCT8, hMCT10 is not labeled with BrAc[^{125}I]T_3 (Fig. 5). Exposure of cells transfected with rD1 cDNA alone to BrAc[^{125}I]T_3 resulted in a minor labeling of the 29-kDa rD1 protein, which was greatly increased by co-transfection with hMCT10 or hMCT8 cDNA. These results indicate that $BrAcT_3$ is transported by hMCT10, resulting in an increased intracellular labeling of rD1, but the transporter itself does not undergo covalent modification by BrAc[^{125}I]T_3. Transfection of cells with hCRYM cDNA alone or together with hMCT10 or hMCT8 cDNA did not result in the obvious labeling of a protein with the expected size of hCRYM (36 kDa), indicating that $BrAcT_3$ does not modify this protein.

Incubation of control transfected COS1 cells with [^{125}I]T_3 in DMEM/F12 medium containing 0.1% BSA resulted in a time-dependent increase in cell-associated radioactivity amounting from 1.1% at 5 min to 2.3% at 30 min (Fig. 6A). In keeping with previous findings, transfection of COS1 cells with hMCT8 cDNA induced a 1.4- to 1.6-fold increase in cellular T_3 uptake. However, transfection of the cells with hMCT10 cDNA produced even larger increases in T_3 uptake, changing from 2.9-fold after 5 min to 2.0-fold after 30 min (Fig. 6A).

The rapid plateauing of the uptake of T_3 by COS1 cells, in particular by cells transfected with hMCT8 or hMCT10 cDNA, suggested that a rapid equilibrium was reached between T_3 influx and efflux. To decrease the rate of T_3 efflux from the cells, their intracellular thyroid hormone-binding capacity was increased by (co)transfection with hCRYM, a cytosolic protein with high affinity for iodothyronines, preferring $T_3 > T_4 > rT_3$ (31–33). Transfection of COS1 cells with hCRYM alone did not affect T_3 uptake (data not shown), but transfection of COS1 cells with either hMCT8 or hMCT10 in addition to hCRYM resulted in a much larger increase

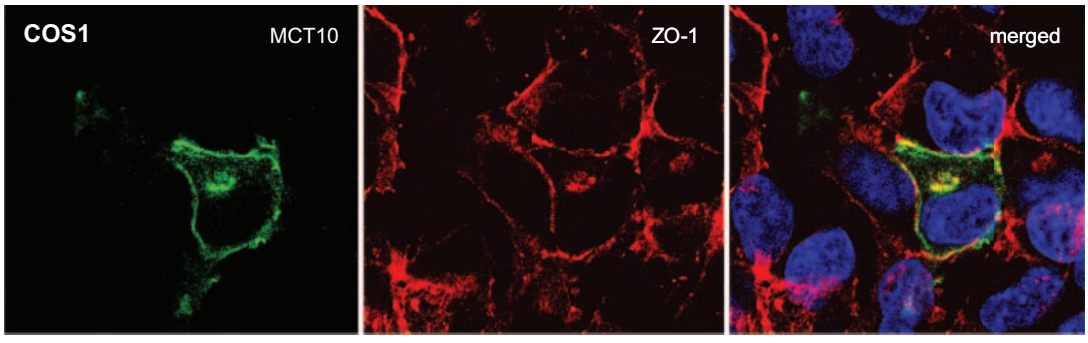

Fig. 4. Immunocytochemistry of COS1 Cells Transfected with hMCT10

Left panel, hMCT10 protein detected with polyclonal antibody 1758 and stained with goat antirabbit Alexa Fluor 488. *Middle panel*, Plasma membrane staining using antibody against tight junction protein ZO-1 and goat antimouse Alexa Fluor 633. *Right panel*, Merged images of hMCT10, plasma membrane marker, and nuclear marker (DAPI staining).

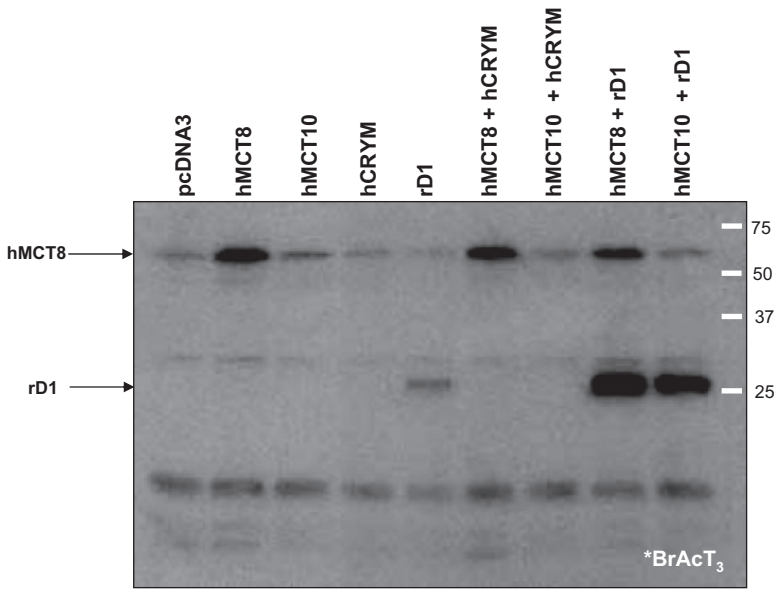

Fig. 5. Affinity-Labeling of hMCT8, hMCT10, hCRYM, and/or rD1 (Co)transfected COS1 Cells with BrAc[^{125}I]T_3

A band of approximately 60 kDa is present in control plasmid-transfected cells. This signal is greatly increased in hMCT8- but not in hMCT10-transfected cells, irrespective of cotransfection with hCRYM or rD1. rD1-transfected cells show affinity labeling of a protein of approximately 30 kDa, the appropriate size for rD1. Cotransfection of hMCT8 and hMCT10 increases labeling of rD1, indicating transport of $BrAcT_3$ by hMCT8 as well as hMCT10.

in T_3 uptake than induced by hMCT8 or hMCT10 alone (Fig. 6C). In the presence of hCRYM, the fold increase in T_3 uptake by hMCT8 varied between 6.5 after 5 min and 4.4 after 30 min. Corresponding values for hMCT10 were 6.9 after 5 min and 5.4 after 30 min.

In the absence of hCRYM, transfection of COS1 cells with hMCT8 or hMCT10 alone resulted in a modest increase in T_4 uptake over control cDNA-transfected cells (data not shown). Although transfection of cells with hCRYM cDNA alone did not affect T_4 uptake (data not shown), cotransfection with hMCT8 led to marked time-dependent increases in T_4 uptake, *i.e*. 5.8-fold after 5 min and 17.9-fold after 30 min (Fig. 6E). The fold increase in T_4 uptake after transfection with hCRYM plus hMCT10 compared with hCRYM alone varied between 1.5 after 5 min and 4.5 after 30 min.

We studied the concentration dependence of T_3 uptake in COS1 cells transfected with hCRYM cDNA without or with hMCT8 or hMCT10 cDNA. Increasing the T_3 concentration from 1 nM to 10 μM resulted in a progressive decrease in the percentage uptake of [^{125}I]T_3 in cells not transfected with transporter as well as in cells transfected with hMCT8 or hMCT10. Irrespective of the expression of hMCT8 or hMCT10, [^{125}I]T_3 uptake showed 50% inhibition at approximately 1 μM T_3 (data not shown). Considering the possible saturation of multiple processes, such as T_3 uptake by endogenous and transfected transporters, T_3 binding to BSA, and T_3 binding to hCRYM, no attempts were made to derive kinetic constants from these data.

The above studies were carried out using DMEM/F12, a culture medium containing high concentrations of compounds, in particular (aromatic) amino acids, which may interfere with the transport of iodothyronines by hMCT8 and hMCT10. Therefore, we compared T_3 and T_4 transport by hMCT8 and hMCT10 in transfected COS1 cells incubated with ligand in Dulbecco's PBS (D-PBS) medium, a simple buffered salt solution with $MgCl_2$ and $CaCl_2$ plus 1 g/liter D-glucose added. Figure 6, B and D, shows T_3 uptake by cells transfected without or with hCRYM, respectively, and Fig. 6F shows results for T_4 uptake in cells transfected with hCRYM.

The findings indicate an increase in T_3 and T_4 uptake if cells were incubated with ligand in D-PBS medium compared with DMEM/F12 irrespective of transfection with hMCT8 or hMCT10 cDNA or with control vector. This suggests that compounds present in DMEM/F12 but not in D-PBS medium do not specifically interfere with iodothyronine transport by hMCT8 or hMCT10.

We have also investigated the influence of BSA on T_3 uptake in COS1 cells. In general, T_3 uptake is 3 times higher in the absence than in the presence of 0.1% BSA, but the fold increase induced by cotransfection with hMCT8 or hMCT10 and hCRYM is 6.4- and 6.9-fold in the presence of 0.1% BSA, respectively, and only 4.1-fold for both hMCT8 and hMCT10 in the absence of BSA (data not shown).

We next investigated whether the increased accumulation of T_3 and T_4 in hCRYM-coexpressing cells transfected with hMCT8 or hMCT10 compared with non-CRYM-expressing cells was indeed caused by a diminished iodothyronine efflux. This was done by loading MCT8 or MCT10-transfected cells for 10 min with [^{125}I]T_3 or [^{125}I]T_4, after which cells were briefly

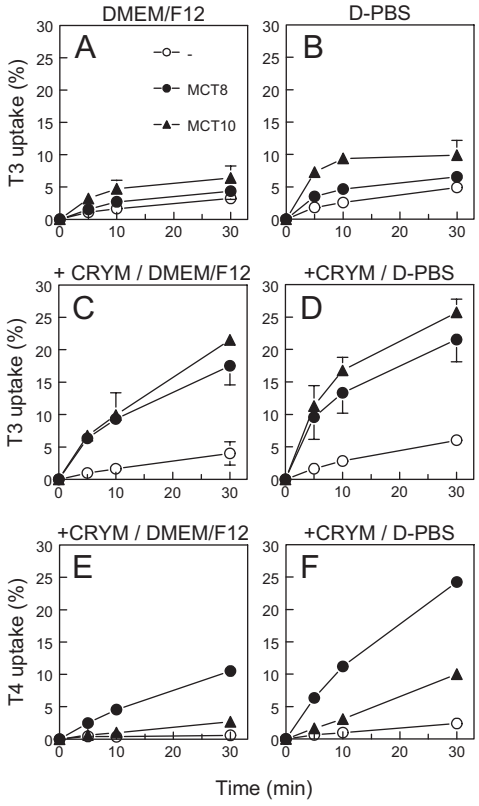

Fig. 6. Uptake of T_3 and T_4 by COS1 Cells Transfected with hMCT8 or hMCT8 cDNA without or with hCRYM cDNA

Cells were transfected with control cDNA (○), hMCT8 cDNA (●) or hMCT10 cDNA (▲) without (A and B) or with (C–F) hCRYM cDNA, and incubated for 5–30 min at 37 C with 1 nM $[^{125}I]T_3$ (A–D) or $[^{125}I]T_4$ (E and F) in DMEM/F12 (A, C, and E) or D-PBS medium (B, D, and F) containing 0.1% BSA. After incubation, cells were processed and cellular radioactivity was determined as described in *Materials and Methods*. Results are presented as means ± SD (n = 2–4).

washed and subsequently incubated for 2, 5, or 10 min with fresh medium without ligand. The results are presented in Figs. 7 and 8, showing a rapid release of most cell-associated T_3 and T_4 in the first 2 min of the efflux incubation. Cellular efflux of T_3 and T_4 appeared faster in cells expressing hMCT8 than in cells expressing hMCT10, which in turn appeared faster than in control cells. Addition of 10 μM unlabeled T_3 (Fig. 7) or T_4 (Fig. 8) to the efflux medium had little effect on the efflux of $[^{125}I]T_3$ or $[^{125}I]T_4$ in the absence of hCRYM.

This rapid release of cellular T_3 and T_4 was largely prevented if cells also expressed hCRYM, indicating that the increased accumulation of T_3 and T_4 in cells expressing hCRYM in addition to hMCT8 or hMCT10 is indeed explained by the increased intracellular binding and, thus, decreased efflux of T_3. The addition of excess unlabeled T_3 or T_4 to the efflux medium greatly increased the rate of $[^{125}I]T_3$ and $[^{125}I]T_4$ efflux from cells cotransfected with hCRYM and hMCT8. Excess medium T_3 also stimulated efflux of $[^{125}I]T_3$ from cells cotransfected with hCRYM and hMCT10. Little efflux of $[^{125}I]T_4$ was observed from cells cotransfected with hCRYM and hMCT10 even in the presence of excess unlabeled T_4 in the medium. Although displacement of $[^{125}I]T_3$ and $[^{125}I]T_4$ from hCRYM and inhibition of their reuptake by excess unlabeled T_3 and T_4 are important contributing mechanisms, the results suggests that efflux is better facilitated by hMCT8 than by hMCT10.

Initially, MCT10 has been characterized as a T-type amino acid transporter primarily using *Xenopus* oocytes as an expression system (11, 12). Using this system, we were unable to detect transport of aromatic amino acids by rMCT8 (15). Therefore, we decided to investigate possible transport of ^{3}H-labeled Phe, Tyr, and Trp by hMCT8 or hMCT10 in transfected COS1 cells. Figure 9 shows a very rapid uptake of all aromatic amino acids in COS1 cells transfected with empty control vector. Because native COS1 cells express MCT10, this background uptake may be explained by endogenous MCT10 but also alternative transporters such as the L-type amino acid transporter that is also capable of transporting aromatic amino acids (35).

Transfection of COS1 cells with hMCT8 did not affect uptake of any of the amino acids, which confirms our previous findings with rMCT8 in oocytes indicating that MCT8 is highly specific for iodothyronines. Surprisingly, transfection of COS1 cells with hMCT10 cDNA resulted in a rapid and large decrease in the accumulation of Phe, Tyr, and Trp by these cells. This suggests that MCT10 primarily mediates the efflux rather than uptake of the different amino acids, which is in agreement with a recent study carried out in *Xenopus* oocytes (36).

Figure 10 shows the effects of 1 mM unlabeled Phe, Tyr, or Trp on the uptake of $[^{125}I]T_3$ by hMCT8 or hMCT10 in COS1 cells cotransfected with hCRYM. Whereas none of the aromatic amino acids inhibit T_3 uptake by hMCT8, Phe, Tyr, and Trp show a 12.5%, 22.2%, and 51.4% inhibition of T_3 uptake by hMCT10, respectively. Addition of 1 mM Trp to the efflux medium had no effect on the efflux of $[^{125}I]T_3$ from cells transfected with hMCT8 (Fig. 11A) or hMCT10 (Fig. 11B) alone or in combination with hCRYM.

We have previously demonstrated that transfection of cells with hMCT8 facilitates intracellular metabolism of different iodothyronines by intracellular D1, D2, and D3 (16). In this study, we addressed the question of whether hMCT10 similarly facilitates intracellular metabolism of T_4 and T_3. Therefore, we transfected COS1 cells with hD3 alone or in combination with hMCT8 or hMCT10, and subsequently incubated the cells for 2 h with $[^{125}I]T_3$ or for 24 h with $[^{125}I]T_4$. In cells transfected with D3 alone, 9% of T_3 was converted to 3,3′-T_2 and 3′-T_1 and 11% of T_4 was converted to rT_3 and further metabolites (Fig. 12). Cotransfection with hMCT8 or hMCT10 resulted in an 8.1- and 8.9-fold increase in the metabolism of T_3 to 70% and 77%, and an 8.8- and 5.0-fold increase in T_4 metabolism to 96% and 54%, respectively. Similar facilitation of intracellular T_4 metabolism was observed in cells transfected with

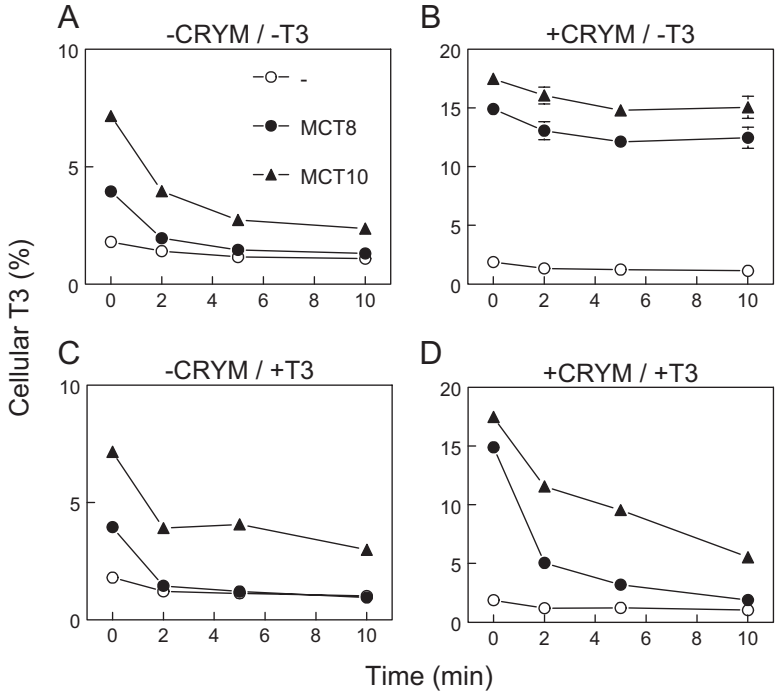

Fig. 7. Effects of hCRYM on Efflux of Cellular T_3

COS1 cells were transfected with control cDNA (○), hMCT8 cDNA (●), or hMCT10 cDNA (▲) without (A and C) or with (B and D) hCRYM cDNA, and incubated for 10 min at 37 C with 1 nM [^{125}I]T_3 in D-PBS medium containing 0.1% BSA. After brief washing, cells were incubated with fresh D-PBS medium plus 0.1% BSA without (A and B) or with (C and D) 10 μM unlabeled T_3. After incubation for 2–10 min at 37 C, cells were processed, and cellular radioactivity was determined as described in *Materials and Methods*. Results are presented as means ± SD (n = 2–4).

hD2 plus hMCT8 or hMCT10 compared with cells transfected with hD2 alone (data not shown).

To demonstrate the physiological importance of hMCT10 for T_3 transport, we performed short interfering RNA (siRNA)-mediated knockdown studies in HEK293 cells. These cells have relatively high endogenous hMCT8 and hMCT10 mRNA expression levels with CT values of 23–24 determined by quantitative PCR (data not shown). HEK293 cells transfected with either one of the three hMCT10-targeted siRNAs showed, after 48 h transfection, a significant 20% reduction of T_3 uptake compared with cells transfected with nonspecific siRNA ($P < 0.0001$; data not shown). Transfection efficiency, as determined using control siRNA labeled with Alexa Fluor 488, amounted to only 25%.

DISCUSSION

Our studies in transfected COS1 cells demonstrate that hMCT10 is an active thyroid hormone transporter. Compared with the homologous hMCT8 thyroid hormone transporter, hMCT10 is more effective in transport of T_3 and less effective in transport of T_4. Both hMCT8 and hMCT10 appear to facilitate bidirectional transport of T_4 and T_3 across the plasma membrane with a net increase in intracellular hormone levels compared with nontransfected cells. This is substantiated by the increase in intracellular iodothyronine metabolism by hD3 if cells are cotransfected with hMCT8 or hMCT10. The efflux of T_3 and T_4 from hMCT8- or hMCT10-expressing cells is strongly diminished if their intracellular binding is increased by expression of the cytosolic binding protein hCRYM, providing a convenient system for studying specifically the uptake of thyroid hormone. In contrast to hMCT8, hMCT10 also transports aromatic amino acids, although it is more effective in the release than in the uptake of these ligands. Given the relatively low transfection level of 25%, the level of inhibition in HEK293 cells observed in our siRNA experiments suggests that hMCT10 is responsible for the majority of T_3 transport in these cells.

MCT10 has been cloned recently from rats and humans and characterized as a T-type amino acid transporter mediating uptake of aromatic amino acids such as Phe, Tyr, Trp, and Dopa (11, 12). However, recent studies indicate that the transporter is much more efficient in facilitating efflux of Phe injected into *Xenopus* oocytes than in facilitating Phe uptake by oocytes (36). Furthermore, the affinity of MCT10 for Phe in both influx and efflux processes is very low, with apparent K_m values exceeding 10 mM (36). In the studies of

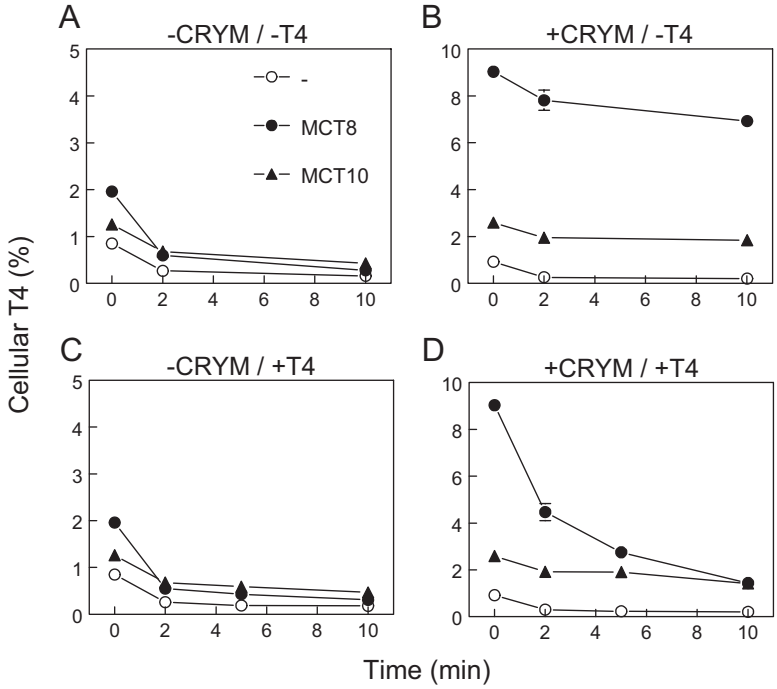

Fig. 8. Effects of hCRYM on Efflux of Cellular T_4

COS1 cells were transfected with control cDNA (○), hMCT8 cDNA (●), or hMCT10 cDNA (▲) without (A and C) or with (B and D) hCRYM cDNA, and incubated for 10 min at 37 C with 1 nM [^{125}I]T_4 in D-PBS medium containing 0.1% BSA. After brief washing, cells were incubated with fresh D-PBS medium plus 0.1% BSA without (A and B) or with (C and D) 10 μM unlabeled T_4. After incubation for 2–10 min at 37 C, cells were processed and cellular radioactivity was determined as described in *Materials and Methods*. Results are presented as means ± SD (n = 2–4).

Endou and co-workers (11, 12), iodothyronine transport by MCT10 could not be detected in oocytes. Furthermore, addition of T_4 and T_3 was found to be without effect on the transport of aromatic amino acids by MCT10.

It is difficult to reconcile the negative findings of the group of Endou with our findings of active transport of iodothyronines, in particular T_3, by hMCT10 that even exceeds T_3 transport by hMCT8. Endou and co-workers (11, 12) used a very high ligand concentration (100 μM) to study T_3 and T_4 uptake by MCT10, which may be supersaturating the transporter and, thus, masking the specific uptake of the labeled iodothyronines. This is supported by our findings that 1 μM T_3 is sufficient to produce 50% saturation of hMCT10. However, the same high concentrations (100 μM) of unlabeled T_3 and T_4 should then be expected to inhibit transport of radioactive amino acids, which was not observed (11,

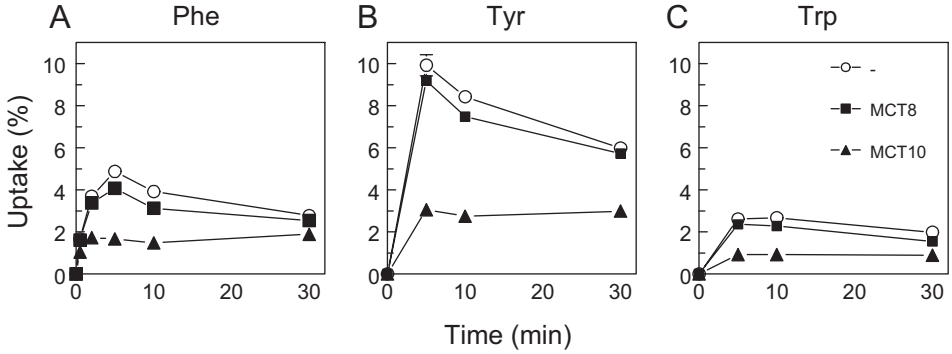

Fig. 9. Uptake of Aromatic Amino Acids by COS1 Cells Transfected with Control cDNA (○), hMCT8 cDNA (●), or hMCT10 cDNA (▲)

Cells were incubated for 0.5–30 min at 37 C with 10 μM [^{3}H]Phe (A), [^{3}H]Tyr (B), or [^{3}H]Trp (C) in D-PBS medium without BSA. After incubation, cells were processed and cellular radioactivity was determined as described in *Materials and Methods*. Results are presented as means ± SD from a representative experiment.

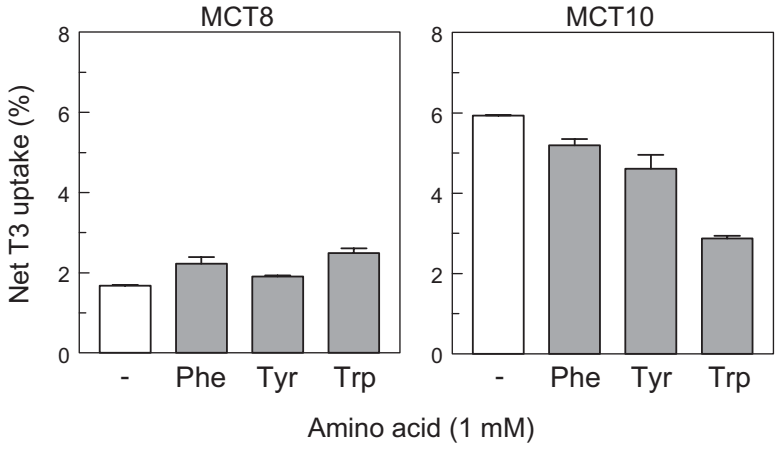

Fig. 10. Influence of Aromatic Amino Acids on the Uptake of T_3

COS1 cells were transfected with hMCT8 (A) or hMCT10 (B) cDNA and incubated for 10 min with 1 nM [^{125}I]T_3 in D-PBS medium containing 0.1% BSA and with or without 1 mM Phe, Tyr, or Trp. Results are presented as uptake minus uptake from control transfected COS1 cells. Results are presented as means ± SD from a representative experiment.

12). This may be explained if iodothyronines and the aromatic amino acids do not share the same binding sites on the transporter or if amino acid influx and efflux are equally affected by iodothyronines. *Vice versa*, we observed a 50% inhibition of hMCT10-mediated T_3 uptake by 1 mM Trp, indicating competition between Trp and T_3 transport by hMCT10. On the other hand, we did not find any effect of Trp on the efflux of cellular T_3.

Of special interest is the lack of affinity labeling of hMCT10 by BrAc[^{125}I]T_3 in contrast to the efficient labeling of hMCT8. Although other amino acids may also be modified, the primary targets for protein labeling by bromoacetylated ligands are the Cys residues. In this regard, it is interesting to note that hMCT8 has 10 Cys residues, most of which are located in putative TMDs. Eight of these Cys residue are also present at corresponding positions in hMCT10, but the Cys residues at positions 423 and 472 in hMCT8 correspond to Phe and Lys in hMCT10, respectively.

We are currently investigating whether one of these Cys residues is the site of BrAcT_3 labeling of hMCT8 and if their absence in hMCT10 explains why this protein is not labeled. The lack of labeling of hMCT10 is not due to a decreased affinity of BrAcT_3 for this transporter, because hMCT10 is at least as efficient as

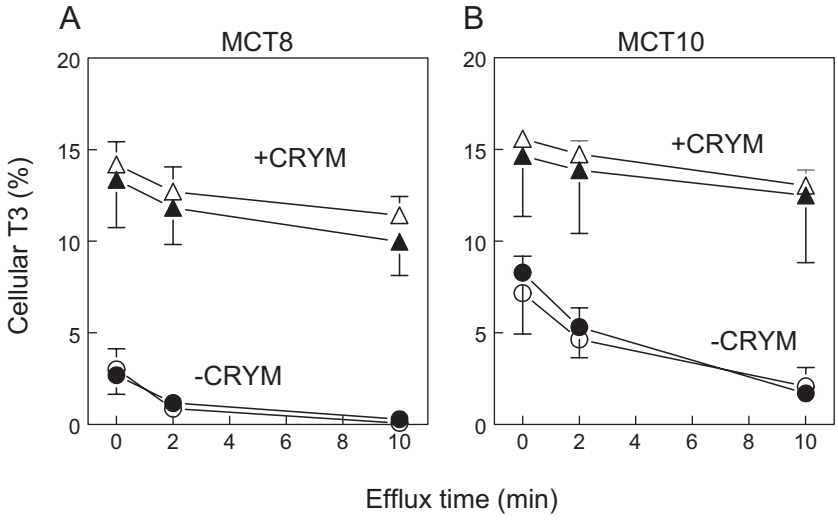

Fig. 11. Influence of Trp on the Efflux of Cellular T_3

COS1 cells were transfected with hMCT8 cDNA (A) or hMCT10 (B) cDNA without or with hCRYM cDNA and incubated for 10 min at 37 C with 1 nM [^{125}I]T_3 in D-PBS medium containing 0.1% BSA. After brief washing, cells were incubated with fresh D-PBS medium plus 0.1% BSA without (*open symbols*) or with (*solid symbols*) 1 mM Trp. After incubation for 2–10 min at 37 C, cells were processed and cellular radioactivity was determined as described in *Materials and Methods*. Results are presented as means ± SD (n = 3).

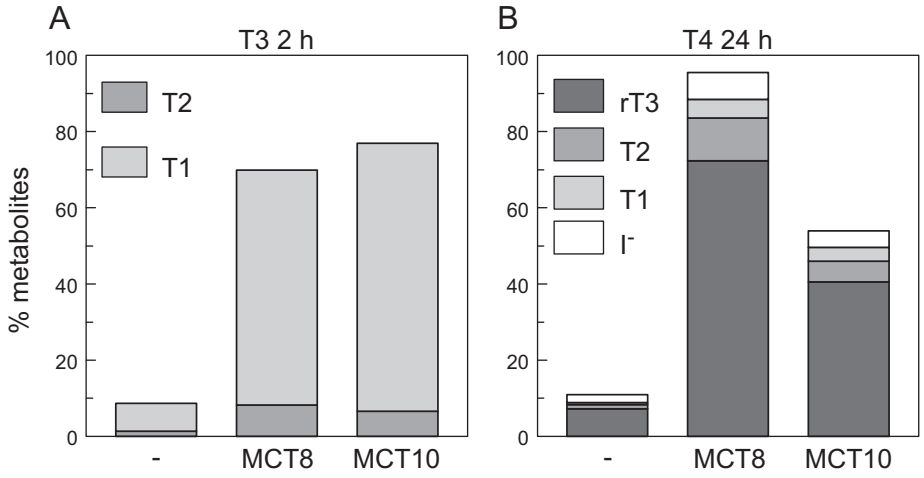

Fig. 12. Effects of hMCT8 and hMCT10 on Intracellular Metabolism of T_3 and T_4 by hD3

COS1 cells were cotransfected with hD3 cDNA and control, hMCT8, or hMCT10 cDNA and incubated for 2 h or 24 h at 37 C with 1 nM [^{125}I]T_3 (A) or [^{125}I]T_4 (B), respectively, in DMEM/F12 medium containing 0.1% BSA. Metabolism of T_3 and T_4 was analyzed by HPLC as described in *Materials and Methods*. Results are the means of duplicate determinations from a representative experiment.

hMCT8 in facilitating the cellular entry of $BrAcT_3$, allowing its labeling of the intracellular rD1 active center. The lack of hMCT10 labeling by $BrAcT_3$ may thus be explained by the absence of a (SH) group susceptible to covalent modification by the bromoacetyl derivative. Within rD1, the selenocysteine residue is thought to be the target for $BrAcT_3$ labeling.

What could be the physiological relevance of MCT10 as a thyroid hormone transporter? Our studies indicate that the transporter facilitates the cellular entry of T_4 and, in particular, T_3, allowing their access to intracellular processes such as metabolism by deiodinases. It is equally likely that MCT10 also facilitates T_3 access to its nuclear receptors. Although this has been clearly demonstrated for the L-type amino acid transporter, which also transports T_3, further studies are required to establish such a role for MCT8 and MCT10 in thyroid hormone action (37).

However, because hMCT8 and hMCT10 mediate bidirectional transport of iodothyronines, they may also be important for the release of T_3 from cells in which the hormone is produced by deiodination of its precursor T_4. In D1-expressing tissues such as liver and kidney, this T_3 is released into the circulation to be transported to its various target tissues (2). In D2-expressing tissues, such as the brain, T_3 is preferentially supplied to local targets (2, 38–40). Such a paracrine regulation of local T_3 supply involves the conversion of T_4 to T_3 in D2-expressing astrocytes and subsequent routing of T_3 to its major target cells, the neurons (38–40). MCT8 appears crucial for T_3 uptake by neurons, and hemizygous mutations of the *MCT8* gene result in severe psychomotor retardation (18–27). In the regulation of local T_3 levels in the brain, T_3 release from astrocytes is an equally important process as T_3 uptake by neurons and T_4 uptake by astrocytes. Equally essential is the transport of T_4 across the blood-brain barrier. Other than the localization of the T_4-specific organic anion transporting polypeptide 1C1 transporter in brain capillaries, nothing is known about this important process (41–43).

The exact cellular and subcellular localization of MCT10 should provide clues about its physiological role. MCT10 mRNA expression has been detected in different tissues, including the entire gastrointestinal tract, liver, kidneys, and skeletal muscle, with relatively low levels of expression in brain (11, 12, 36). In kidney, MCT10 protein was located in the basolateral membranes of proximal tubular cells, in liver it was localized to the basolateral membrane of centrilobular hepatocytes, and in the intestine a basolateral localization was observed in entrocytes increasing toward the tip of the villi (36). In liver and kidney, MCT10 may be involved in plasma↔tissue transfer of thyroid hormone, and it is tempting to speculate that in the intestine MCT10 may be important for thyroid hormone absorption.

Particularly interesting is the high expression of MCT10 mRNA in placenta (see expression profile in GenBank http://www.ncbi.nlm.nih.gov/UniGene/EST-Profile Viewer.cgi?uglist=Hs.591327). Placental transfer of maternal thyroid hormone is essential for fetal development, in particular of the brain (44). This is especially important during the first trimester of gestation when the fetal thyroid has not yet developed. Subsequently, there is an increasing contribution of the fetal thyroid to circulating hormone levels, although in case of defective thyroid development or thyroid hormone synthesis, maternal thyroid hormone remains an important source for the fetus throughout gestation (44). Possibly, MCT10 is involved in the placental transfer of maternal thyroid hormone to the fetus. However, fetal brain development depends on the supply of T_4 as a substrate for local T_3 generation

rather than on supply of circulating T_3 (2, 39, 44). Placental transport may also be greater for T_4 than for T_3, which argues against an important role for MCT10 that prefers T_3 over T_4 as the ligand. However, maternal-fetal transfer of thyroid hormone may well be controlled, to a large extent, by the high placental expression of D3, which is more effective in the degradation of T_3 than of T_4 (45, 46). It is important to investigate the involvement of MCT10 in the placental transfer of thyroid hormone.

In conclusion, our studies clearly demonstrate that MCT10 is a thyroid hormone transporter with preference for T_3 over T_4. It appears an even more effective T_3 transporter than MCT8, which is likely to play a crucial role in neuronal T_3 supply in the brain. The importance of MCT10 for cellular entry or efflux of T_3 in different tissues remains to be fully explored.

MATERIALS AND METHODS

Materials

Nonradioactive iodothyronines and aromatic amino acids were obtained from Henning (Berlin, Germany) or Sigma Chemical Co. (St. Louis, MO). [3′-^{125}I]T_3 and [3′,5′-^{125}I]T_4 (1500–2000 mCi/μmol), [3,5-^{3}H]Tyr (52 Ci/mmol), [5-^{3}H]Trp (30 Ci/mmol), and [2,3,4,5,6-^{3}H]Phe (116 Ci/mmol) were obtained from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK). Radioactive *N*-bromoacetyl-T_3 (BrAc[^{125}I]T_3) was synthesized as previously described (34). FuGENE6 transfection reagent was obtained from Roche Diagnostics (Almere, The Netherlands). The pcDNA3 and pcDNA3.1 expression vectors were obtained from Invitrogen (Breda, The Netherlands), and pSG5 was obtained from Stratagene (Amsterdam, The Netherlands).

Plasmids

Cloning of hMCT8 cDNA in the pcDNA3 expression vector has been described previously (16). IMAGE clones of full-length hMCT10 cDNA and hCRYM cDNA were obtained from the RZPD German Resource Center for Genome Research (www.rzpd.de). hMCT10 cDNA was subcloned into the expression vector pcDNA3.1 using *Eco*RI and *Xba*I restriction sites, and hCRYM cDNA was subcloned into pSG5 using *Eco*RI and *Bam*HI. Expression vectors containing rat type I deiodinase cDNA (pcDNA3.rD1) or human type III deiodinase cDNA (pCIneo.hD3) were obtained as previously described (16).

Cell Culture

COS1 cells were cultured in six- or 24-well dishes (Corning, Schiphol, The Netherlands) with DMEM/F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (Invitrogen) and 100 nM sodium selenite (Sigma). Cells were cultured 24 h after transfection.

Immunoblotting and Immunocytochemistry

Polyclonal antisera were raised in rabbits by Eurogentec SA (Seraing, Belgium) against synthetic peptides comprising amino acids 473–487 and 503–515 of hMCT10 conjugated to keyhole limpet hemocyanin. Antiserum (no. 1758) from the final bleed was used after IgG purification (Eurogentec). IgG-purified hMCT8 antibody 1306 was obtained as previously described (16).

hMCT10 and hMCT8 proteins were expressed in COS1 cells cultured in six-well plates by transfection with 500 ng pcDNA3.1.hMCT10 or pcDNA3.hMCT8 with 500 ng pcDNA3 or 500 ng pSG5.hCRYM using 3 μl FuGENE6 transfection reagent. Empty pcDNA3 or hCRYM alone was used as a control. After 24 h, the cells were rinsed with PBS and collected in 200 μl 0.1 M phosphate buffer (pH 7.2) and 2 mM EDTA (P100E2). The cells were sonicated on ice, aliquoted and stored at −80 C. Homogenates (10–15 μg protein) were separated on 12% SDS-PAGE minigels. Thereafter, the proteins were blotted on nitrocellulose membranes, probed with antiserum 1758 or 1306 (1:1000), and further processed as described previously (47).

COS1 cells were cultured on 15-mm glass coverslips coated with poly-D-lysine. After 24 h, cells were transfected with 400 ng hMCT10 cDNA using 1.2 μl FuGENE6. After 24 h, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 5 min. Samples were blocked in PBS containing 2% BSA for 30 min and stained with polyclonal rabbit anti-MCT10 antibody 1758 (1:1000) and monoclonal mouse anti-zona occludens 1 (ZO-1) antibody (Invitrogen) (1:250). After secondary staining with goat antirabbit Alexa Fluor 488 and goat antimouse Alexa Fluor 633 (Invitrogen) (1:250), coverslips were mounted with Prolong Gold containing DAPI for nuclear staining (Invitrogen). Samples were examined on a Zeiss Axiovert 100 confocal microscope using Zeiss LSM software (Carl Zeiss, Sliedrecht, The Netherlands).

Affinity Labeling of Transfected Proteins with BrAcT_3

COS1 cells grown in six-well plates were cotransfected in duplicate with either 1000 ng empty pcDNA3 or 500 ng pcDNA3.1.hMCT10 or pcDNA3.hMCT8 plus 500 ng empty pcDNA3, pcDNA3.rD1, or pSG5.hCRYM using 3 μl FuGENE6. After 24 h, the cells were washed with serum-free DMEM/F12 medium and incubated for 4 h at 37 C with 400,000 cpm BrAc[^{125}I]T_3 in 1.5 ml serum-free DMEM/F12 medium per well. The cells were washed with PBS, and duplicate wells were pooled and lysed in 200 μl SDS-PAGE loading buffer containing 10 mM dithiothreitol and sonicated on ice. The samples were analyzed by SDS-PAGE (12% gels), followed by autoradiography to BioMax MS film (Eastman Kodak Co., Rochester, NY) at −80 C with intensifying screen (2–10 d exposure).

Thyroid Hormone Uptake and Efflux Experiments

COS1 cells were cultured in six-well dishes, and cotransfected in duplicate as described for immunoblotting. After 24 h, cells were washed with DMEM/F12 or D-PBS medium containing 0.1% BSA and incubated for 5–30 min at 37 C with 1 nM (2×10^5 cpm) [^{125}I]T_4 or [^{125}I]T_3 in 1.5 ml DMEM/F12 or D-PBS medium plus 0.1% BSA. To study the effects of aromatic amino acids, we incubated (transfected) COS1 cells with [^{125}I]T_3 in the absence or presence of 1 mM unlabeled Phe, Trp, or Tyr. After incubation, cells were washed with medium with 0.1% BSA, lysed with 0.1 M NaOH, and counted. For efflux studies, COS1 cells were loaded for 10 min with 1 nM (2×10^5 cpm) [^{125}I]T_4 or [^{125}I]T_3 in D-PBS, briefly washed, and subsequently incubated for 2–10 min with 1.5 ml fresh medium with 0.1% BSA without radioactive ligand and with or without excess unlabeled T_3 or T_4 or Trp. After incubation, medium and cells were collected without further washing.

Amino Acid Uptake

COS1 cells were cultured in six-well dishes and transfected in duplicate with 500 ng empty pcDNA3, pcDNA3.1.hMCT10, or pcDNA3.hMCT8. After 24 h, cells were washed with D-PBS without BSA and incubated for 0.5–30 min at 37 C with 10 μM [^{3}H]Phe, [^{3}H]Tyr, or [^{3}H]Trp in 1.5 ml D-PBS. After incubation, cells were washed, lysed with 0.5% SDS, and counted.

Iodothyronine Metabolism Experiments

COS1 cells were cultured in 24-well culture dishes and transfected in duplicate with 200 ng empty pcDNA3, 100 ng pcDNA3.1.hMCT10, or pcDNA3.hMCT8 plus 100 ng empty pcDNA3 or pCIneo.hD3 using 0.6 μl FuGENE 6. After 24 h, cells were washed with DMEM/F12 plus 0.1% BSA and incubated for 2–24 h at 37 C with 1 nM (1×10^6 cpm) [^{125}I]T_4 or [^{125}I]T_3 in 0.5 ml DMEM/F12 plus 0.1% BSA. After incubation, medium was sampled, processed, and analyzed by HPLC as previously described (16). More than 80% of added radioactivity was recovered from the medium, and recovery of injected radioactivity over the HPLC was almost 100%.

MCT10 Knockdown Experiments

HEK293 cells were transfected with three different HP GenomeWide siRNAs against hMCT10 (QIAGEN, Venlo, The Netherlands), *i.e.* Hs_SLC16A10_2_HP siRNA with target sequence CACAATAATTGGGAAATAGAA located at the beginning of the 3′-untranslated region, Hs_SLC16A10_4_HP siRNA with target sequence CACGTTTCTGAATTTGTTTAA located at the end of the 3′-untranslated region, and Hs_SLC16A10_5_HP siRNA with target sequence TACCTTACCTATGGAATCATA located in the coding region. Control transfections were performed with a nonsilencing control siRNA labeled with Alexa Fluor 488 allowing easy monitoring of transfection efficiency. Transfection was performed according to the manufacturer's protocol using HiPerfect Transfection Reagent (QIAGEN) and 20 nM siRNA. After 48 h transfection, HEK cells were incubated for 5 min with [^{125}I]T_3 as described above.

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