Disruption of transport activity in a D93H mutant thiamine transporter 1, from a Rogers Syndrome family

Dana Baron¹, Yehuda G. Assaraf², Stavit Drori² and Ami Aronheim¹

¹Department of Molecular Genetics, The Rappaport Institute for Research in the Medical Sciences and the B. Rappaport Faculty of Medicine, and ²Department of Biology, The Technion-Israel Institute of Technology, Haifa, Israel

Rogers syndrome is an autosomal recessive disorder resulting in megaloblastic anemia, diabetes mellitus, and sensorineural deafness. The gene associated with this disease encodes for thiamine transporter 1 (THTR1), a member of the SLC19 solute carrier family including THTR2 and the reduced folate carrier (RFC). Using transient transfections into NIH3T3 cells of a D93H mutant THTR1 derived from a Rogers syndrome family, we determined the expression, post-translational modification, plasma membrane targeting and thiamine transport activity. We also explored the impact on methotrexate (MTX) transport activity of a homologous missense D88H mutation in the human RFC, a close homologue of THTR1. Western blot analysis revealed that the D93H mutant THTR1 was normally expressed and underwent a complete N-glycosylation. However, while this mutant THTR1 was targeted to the plasma membrane, it was completely devoid of thiamine transport activity. Consistently, introduction into MTX transport null cells of a homologous D88H mutation in the hRFC did not result in restoration of MTX transport activity, thereby suggesting that D88 is an essential residue for MTX transport activity. These results suggest that the D93H mutation does not interfere with transporter expression, glycosylation and plasma membrane targeting. However, the substitution of this negatively charged amino acid (Asp93) by a positively charged residue (His) in an extremely conserved region (the border of transmembrane domain 2/intracellular loop 2) in the SLC19 family, presumably inflicts deleterious structural alterations that abolish thiamine binding and/or translocation. Hence, this functional characterization of the D93H mutation provides a molecular basis for Rogers syndrome.

Keywords: Rogers syndrome; thiamine; transporter; mutations.

Thiamine responsive megaloblastic anemia (TRMA) also known as Rogers syndrome [1], is an early onset autosomal recessive disorder that was described in only 20 families all over the world. Patients with Rogers syndrome are diagnosed by the occurrence of multiple clinical manifestations including: megaloblastic anemia, diabetes mellitus and sensorineural deafness [Online Mendelian Inheritance in Man (OMIM) 249270, http://www.ncbi.nlm.nih.gov/Omim/]. The administration of high doses of thiamine or alternatively the use of medications such as sulfonlureas recovers insulin secretion [2–4]. However, with disease progression, insulin secretion gradually declines to levels that require insulin therapy. On the other hand, in all TRMA patients, anemia is fully reversed following the administration of high doses of thiamine [5].

Fibroblasts isolated from Rogers syndrome patients, display only ~5–10% of thiamine uptake as compared with fibroblasts derived from healthy individuals. This uptake apparently occurs via a low-affinity, unsaturable thiamine route [6]. These results are consistent with an entry of thiamine via passive diffusion and may explain the fact that high doses of thiamine are able to induce minimally sufficient thiamine levels in Rogers syndrome patients.

The gene responsible for Rogers syndrome, SLC19A2, was identified by positional cloning [7–9]. SLC19A2 encodes for a thiamine transporter (THTR1), a member of the solute carrier family. This family includes SLC19A1 that encodes for the reduced folate carrier (RFC) and SLC19A3 that encodes for thiamine transporter 2, THTR2 [10]. THTR1 contains 497-amo acids and has 12 putative transmembrane domains (TMD) [7–9]. THTR1 is responsible for thiamine transport in a Na⁺ independent manner and is stimulated by pH⁺ efflux gradient [11].

Although all Rogers syndrome patients exhibit similar clinical manifestations, multiple mutations were identified resulting in premature translation termination due to base pair insertions or deletions which result in frame-shift. In addition, three missense mutations were described resulting in single amino-acid substitutions. Recently we have initiated
studies that are aimed at characterization of the impact that these missense \textit{THTR1} mutations have on the expression, post-translational modification, plasma membrane targeting and thiamine transport activity. In a recent paper, we described the molecular consequences of a single amino-acid substitution identified in an Italian Rogers syndrome family harboring a glycine to aspartate substitution at position 172 of the \textit{THTR1} [12]. Although this mutant \textit{THTR1} gene is properly transcribed and translated \textit{in vitro}, no protein could be detected in NIH3T3 cells transfected with the mutant G172D \textit{THTR1} cultured at 37 °C. Shifting transfected cells to 28 °C resulted in a substantial expression of the mutant G172D \textit{THTR1} protein thereby allowing for biochemical and functional characterization [12]. The G172D mutant \textit{THTR1} failed to undergo a complete N-glycosylation, on the glutamine 63 acceptor which is found to be conserved in both \textit{THTR1} and \textit{THTR2} [12] resulting in its cytoplasmic retention in the endoplasmic reticulum. This is in contrast to the other members of the solute carrier family, including the hRFC, in which N-glycosylation plays neither a role in its plasma membrane localization nor in MTX transport activity [13]. Here we describe the characterization of another mutation identified in a French Rogers family harboring an aspartate to histidine substitution at position 93 (D93H) in \textit{THTR1} [14]. Unlike in a previous analysis of the D93H mutation [15], here we show that the mutant protein was efficiently expressed both \textit{in vitro} and \textit{in vivo} and upon immunofluorescence studies this protein was properly targeted to the plasma membrane. Uptake studies with the D93H mutant protein revealed no \textit{[^{3}H]thiamine transport activity. Importantly, this D93 region in \textit{THTR1} and the homologous D88 vicinity in the hRFC are extremely conserved among all members of the solute carrier family (SLC19A1, SLC19A2 and SLC19A3). Indeed, a hRFC harboring the same D88H mutation displayed no methotrexate transport activity. This is consistent with a previous report in which a D88V mutant RFC showed no transport activity [16]. We suggest that this site (i.e. D93 in \textit{THTR1} and D88 in hRFC) represents a highly conserved region, which is absolutely essential for the structure and function of all members of the solute carrier family. Hence, the present study constitutes the first demonstration of a \textit{THTR1} mutation that does not interfere with transporter expression, N-linked glycosylation and plasma membrane targeting but completely disrupts thiamine transport activity.

\section*{Experimental procedures}

\subsection*{Cell cultures}

NIH3T3 and Swiss 3T3 cells were cultured under monolayer conditions in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/mL penicillin and 100 µg/mL streptomycin.

\subsection*{Transient and stable transfections}

Transient NIH3T3 cell transfections were carried out in 60 mm plates using the LipofectAMINE reagent (Gibco-BRL Inc.), whereas XtremeGENE reagent (Roche Inc.) was used for Swiss 3T3 cells. Typically, for NIH3T3 cell transfections, 6 µg of plasmid DNA were used with 9 µL of the LipofectAMINE reagent whereas for Swiss 3T3 cells, 2 µg of plasmid DNA were used along with 10 µL of XtremeGENE reagent according to the instructions of the manufacturer.

\subsection*{Cell extract}

Whole cell extracts were isolated as previously described [17].

\subsection*{Isolation of \textit{THTR1} cDNA}

\textit{THTR1} cDNA was isolated as previously described [12].

\subsection*{Mammalian expression plasmids}

pCan-\textit{THTR1} expression plasmids; pCan is a pcDNA-based (Invitrogen Inc.) mammalian expression vector expressing proteins downstream of a Myc-epitope tag. Protein expression is under the control of cytomegalovirus (CMV) immediate early promoter. An oligonucleotide based PCR approach was used to amplify the human \textit{THTR1} cDNA that was fused, in frame, with the Myc-epitope tag using 5'-EcoRI and 3'-XhoI restriction sites. The Myc tag and polylinker regions are composed of the following 17 amino acids: MVQKLISEEDLRIHRCR. For fusion of Myc tag to the C-terminus of the \textit{THTR1}, an oligonucleotide-based PCR approach was used to amplify the human \textit{THTR1} cDNA that was fused in frame, with the Myc-epitope tag using 5'-HindIII and 3'-HindIII restriction sites with the Myc tag in the 3'-end. The correct orientation was verified by XhoI digestion generating a 1100-bp fragment due to a unique XhoI site at position 377 within \textit{THTR1} cDNA and at the 3'-end of the pCan expression vector’s multiple cloning site.

\subsection*{GFP expression plasmid}

This plasmid encodes for the green fluorescent protein under the control of the CMV promoter (Life Technologies Inc.).

\subsection*{Site-directed mutagenesis}

The corresponding oligonucleotides were designed to yield an aspartate 93 to histidine substitution and to generate a diagnostic \textit{AatII} restriction site. Site directed mutagenesis was performed using the QuickChange XL site directed mutagenesis kit according to the instructions of the manufacturer (Stratagene Inc.) Primers used for mutagenesis of \textit{THTR1} (D93H) were: Upstream oligo: 5'-CCCTGTTTTCCCTGCGACACACTACCTCGGTTA TAAACC-3' and downstream oligo: 5'-GGTATTAAAAAG GAGGTAGTGTGGCAAGGACAGCAGG-3'. DNA sequencing was used to verify that \textit{THTR1} was free of any other mutations. The primers for the introduction of the D88H mutation in the hRFC were: Upstream oligo: 5'-GTTTTCTGCTCACCCACTAAGTCCGAC-3' and downstream oligo: 5'-CGTGAGCGCAGGT AGTGAGTGGAGGAGAAGCCAC-3'.
**In vitro translation studies**

Coupled *in vitro* transcription and translation were performed using the T7 TNT quick transcription and translation kit according to the instructions of the manufacturer (Promega Inc.).

**Tunicamycin treatment**

Tunicamycin (Sigma Cat. # T7765) was dissolved in dimethyl sulfoxide to yield a stock solution of 10 mg·mL⁻¹. This N-glycosylation inhibitor was applied to monolayer cells at a final concentration of 10 μg·mL⁻¹ for 24 h prior to cell harvesting and immunofluorescent staining.

**Peptide N-glycosidase F (PNGaseF) treatment**

Aliquots of cell extracts (50 μg) were incubated with a denaturing buffer containing sodium phosphate pH 7.5, 0.5% SDS and 1% 2-mercaptoethanol in the presence or absence of 2000 U of PNGaseF (NEB Inc.) for 45 min at 37 °C.

**SDS/PAGE and Western blotting**

Aliquots of protein extracts (50 μg) or 5 μL of a programmed rabbit reticulocyte lysate were resolved on a 10% SDS/PAGE followed by immobilization onto Hybond nitrocellulose membrane (Amersham Inc.). Western blots were then blocked with a buffer containing: 10% low fat milk in NaCl/Pi for 1 h and incubated with mouse anti-Myc 9E11 monoclonal IgG (Babco Ltd) followed by a secondary goat anti-(mouse Ig) Ig conjugated to horse radish peroxidase (HRP). Detection was performed with a chemiluminescent substrate (SuperSignal, Pierce Inc.) and membranes were exposed to X-ray films and autoradiogram with linear exposures were obtained.

**Immunofluorescence**

Swiss 3T3 cells were grown on 22-mm coverslips and transfected as described above. Forty-eight hours after transfection, cells were fixed and immunostained as described [12]. Each coverslip contained at least 5 × 10⁴ cells and the efficiency of transfection varied between 10 and 20%. About 90% of transfected cells exhibited red and green staining and all of these cells displayed the staining pattern. At least 100 transfected cells per slide from each transfection were examined. The immunofluorescence analysis, which was reproduced in five independent transfection experiments, revealed an identical staining pattern.

**Assay of thiamine uptake**

NIH3T3 cells transiently transfected with wild-type and mutant D93H THTR1 and vector control were detached with NaCl/Pi containing 1 mM EDTA 2 h prior to thiamine transport measurements. Cells were incubated in a 15-mL tube (Greiner Ltd) at a density of 3 × 10⁶ cells·mL⁻¹ in serum-free DMEM lacking thiamine. NIH3T3 cells were centrifuged and resealed in 100 μL of transport buffer containing 0.6 μM of [³H]thiamine (30 Ci·mmol⁻¹; American) for 20 min at 37 °C as previously described [11]. Cells were then washed twice with 10 mL of ice-cold NaCl/Pi and lysed with 0.2 mL of 0.2 M NaOH, 0.1% SDS at 65 °C for 30 min [18]. The lysate was used to determine intracellular radioactivity in a liquid scintillation spectrometer.

**Stable transfections with wild-type and D88H mutant hRFC cDNA**

A wild-type hRFC cDNA clone containing the entire coding region as well as a D88 mutant RFC cDNA prepared by site-directed mutagenesis (QuickChange XL kit, Stratagene) were directionally cloned into pcDNA3 at the BamHI/XhoI site (Invitrogen). Mouse leukemia cells, L1210 and their MTX transport null cells, MTX/A, were used for transfections. Exponentially growing cells (2 × 10⁶) were harvested by centrifugation and stably transfected by electroporation (1000 μF, 234 V) with 10 μg of an expression vector (pcDNA3-hRFC1) harboring the normal hRFC or the D88H mutant hRFC cDNA. Following 24 h of growth at 37 °C, cells were exposed to 600–750 μg·mL⁻¹ active G-418 (Calbiochem-Novabiochem, San Diego, CA). Cells were then distributed into 96-well plates at ≥ 2 × 10⁴–4 × 10⁴ cells per well and after 10–20 days of incubation at 37 °C, individual G418-resistant clones were picked and expanded for further studies. Only clones expressing comparable RFC mRNA levels were used.

**Assay of MTX transport**

Antifolate transport measurements were performed as previously described [19]. Briefly, exponentially growing cells were harvested, washed twice with Hepes/NaCl/Pi at pH 7.4 and suspended at a density of 2 × 10⁵ cells·mL⁻¹ at 37 °C. The uptake of [³H]MTX (specific activity 0.5 Ci mmol⁻¹) was measured at an extracellular concentration of 2 μM in 1 mL of cell suspension at 37 °C. Influx measurements were performed at 0.5, 1 and 2 min during which MTX transport rates were linear. Uptake was terminated by adding 10 mL of ice-cold Hepes/NaCl/Pi, following which centrifugation and cell wash with another 10 mL ice-cold Hepes/NaCl/Pi was performed. The final cell pellet was processed for liquid scintillation counting.

**Results**

**Expression of THTR1 and D93H THTR1 in NIH3T3 cells**

A Rogers syndrome patient from a French family was found to harbor an aspartate 93 to histidine substitution (D93H) in THTR1. In order to study the effect of this mutation on THTR1 expression and function, we used site-directed mutagenesis to express D93H mutant THTR1 protein fused to an N-terminal Myc tag epitope. NIH3T3 cells were transiently transfected with expression plasmids encoding for either the wild-type or the D93H mutant THTR1. Cell lysates isolated from transfected cells were subjected to SDS/PAGE followed by Western blot analysis using anti-Myc 9E11 monoclonal Igs (Fig. 1A). A strong signal represented by a broadly migrating THTR1 protein with an average molecular mass of 68 kDa was detected in cell lysates derived from both the wild-type and D93H...
mutant THTR1 transfected cells. As expected, no THTR1 was observed in cell lysates derived from vector transfected cells (Fig. 1A). To test the effect of the Myc epitope tag fused to the N-terminal of the protein we also designed expression plasmids encoding for THTR1 fused to a Myc epitope at the C-terminus. Western blot analysis performed with cell lysates derived from NIH3T3 cells transfected with expression plasmids encoding for either the wild-type THTR1 or the D93H mutant THTR1 fused to Myc tag epitope at the C-terminus did not reveal any expression when probed with the anti-Myc Ig (Fig. 1B). To confirm whether THTR1-Myc and D93H mutant THTR1-Myc can be properly transcribed and translated, we used an in vitro system of coupled transcription and translation (rabbit reticulocyte lysate) in the presence of \[^{35}S\]methionine. In vitro translated, radiolabeled proteins were then resolved on SDS/PAGE followed by autoradiography (C) or Western blotting (D).

The mutant D93H THTR1 is N-glycosylated

The broad migration of the THTR1 proteins (Fig. 1A) suggested that the well expressed D93H mutant THTR1 is indeed due to N-glycosylation, we treated transfected NIH3T3 cells with tunicamycin (10 μg/mL), an established N-glycosylation inhibitor [12]. Whole cell extracts derived from mutant D93H THTR1 transfected cells treated with tunicamycin exhibited the same migration pattern on SDS/PAGE when compared to cells transfected with the wild-type THTR1 after treatment with tunicamycin (Fig. 2, compare lane 2 and 5). In order to assess whether N-glycosylation is the post-translational modification responsible for the markedly decreased electrophoretic mobility of the transfected wild-type and D93H mutant THTR1 proteins, we used peptide N-glycosidase F (PNGaseF). PNGaseF is an endoglycosidase, which
cleaves the covalent bond between the innermost N-acetyl-
-N-glucosamine and the acceptor asparagine, thereby resulting in the complete removal of the N-glycan from glycosylated
proteins. Treatment of total cell extracts from transfected cells with PNGaseF resulted in an almost complete removal of the carbohydrate, thereby revealing a sharp 52 kDa protein band with a similar electrophoretic mobility for both the core wild-type THTR1 protein and the in vitro translated THTR1 or the mutant D93H THTR1 proteins (Fig. 2). Thus, these results strongly suggest that the slower migration of both the wild-type and mutant D93H THTR1 proteins is largely due to an N-linked glycosylation. These results suggest that the D93H mutant THTR1 protein is efficiently transcribed, translated and apparently undergoes a complete N-glycosylation as compared to the wild-type THTR1 protein.

Mutant D93H THTR1 is targeted to the plasma membrane

We have previously shown that only the properly folded THTR1 protein is targeted to the plasma membrane and that only the fully glycosylated mature THTR1 protein reaches its plasma membrane destination [12]. We determined whether or not the D93H mutant THTR1 is indeed properly glycosylated such that it will be properly trafficked to the plasma membrane. We used immunofluorescence analysis with Swiss 3T3 cells in order to explore THTR1 protein localization. We employed Swiss 3T3 cells for the immunofluorescence analysis as these cells are larger in size and exhibit a distinct cell morphology as compared with NIH3T3 cells. Swiss 3T3 cells were cotransfected with expression plasmids encoding for wild-type or D93H mutant THTR1 along with an expression plasmid encoding for green fluorescence protein (GFP) (Fig. 3). The GFP expression plasmid is expected to cosegregate with THTR1 or mutant D93H THTR1 expression plasmids. Therefore, cells that exhibit green fluorescence in all cell compartments assist in the localization of the wild-type or D93H mutant THTR1 proteins (Fig. 3A). Wild-type and D93H mutant THTR1 proteins were stained with anti-Myc 9E11 monoclonal Ig followed by CY3-conjugated goat anti-mouse IgG as a second antibody (Fig. 3B). Co-staining with both green (GFP) and red fluorescence (THTR1) is shown (Fig. 3C). Thus, colocalization is represented by a yellow stain.

Cells transfected with THTR1 expression plasmids exhibit perinuclear-endoplasmic reticulum (ER) staining, as well as cytoplasmatic and plasma membrane staining as previously reported (Fig. 3, THTR1) [12]. Similarly, the immunofluorescent localization pattern obtained with the D93H mutant THTR1 was indistinguishable from that observed with the wild-type THTR1 (Fig. 3 compare D93H and wild-type THTR1).

To test whether the glycosylation of the mutant D93H THTR1 plays a role in the plasma membrane localization, as compared with the wild-type THTR1, we treated transfected cells with tunicamycin (10 μg/mL), 24 h prior to immunofluorescence analysis. As previously reported, treatment with tunicamycin resulted in a complete loss of plasma membrane localization of the THTR1 protein (Fig. 3, THTR1 ± tunicamycin) [12]. Cells transfected with the D93H mutant THTR1 and treated with tunicamycin displayed a similar lack of plasma membrane localization of the mutant transporter as well as a typical subcellular localization that was confined to the perinuclear ER membrane (Fig. 3, D93H ± tunicamycin).

Transport in cells transfected with THTR1 or D93H THTR1

The fact that the D93H mutant THTR1 protein is expressed, fully glycosylated and sorted out to the plasma membrane as does the wild-type THTR1 protein raised the question of the functionality (i.e. thiamine transport activity) of this mutant THTR1. To test the ability of the mutant THTR1 to transport thiamine, we transiently transfected NIH3T3 cells with expression plasmids representing the empty vector as well as expression plasmids encoding for either the wild-type THTR1 or the D93H mutant THTR1 and measured [3H]thiamine uptake. NIH3T3 cells transfected with an empty expression vector exhibited an endogenous background thiamine transport activity (Fig. 4). Expectantly, NIH3T3 cells transfected with an expression vector harboring the wild-type THTR1 exhibited a 2.5-fold increase in thiamine uptake as compared with cells transfected with the empty expression vector. In contrast, cells transfected with the D93H mutant THTR1 showed thiamine uptake reflecting the background levels that were obtained with cells transfected with the empty vector (Fig. 4) or untransfected cells (data not shown). These results establish that the mutant D93H THTR1 protein lacks thiamine transport activity. Furthermore, this finding is in accord with Rogers syndrome patients carrying this D93H mutant protein which apparently lost thiamine transport activity.

Aspartate 93 is conserved in all members of the solute carrier SLC19 family

Aspartate 93 is located in the border of TMD2 and the beginning of intracellular loop 2 that is composed of only eight amino acids [11]. This region is extremely conserved in
Fig. 3. Sub-cellular localization of D93H mutant THTR1 proteins by immunofluorescence. Swiss 3T3 cells were cotransfected with a GFP expression plasmid, with the Myc-THTR1 or mutant D93H THTR1 expression plasmids as indicated. Cells were treated (where indicated) with 10 µg·mL⁻¹ tunicamycin for 24 h prior to cell fixation and staining with anti-Myc 9E11 monoclonal Ig and secondary goat-anti-(mouse Cy3 Ig) Ig. Cells were then analyzed using confocal microscopy. The localization of GFP alone (green, A) or Cy3-conjugated goat-anti-(mouse Ig) Ig staining alone (red, B) and costaining (orange/yellow, Panel C) is shown. Orange/yellow stain indicates colocalization. Cell transfections and treatments were as indicated: Wild-type THTR1 (THTR) and mutant D93H THTR1 (D93H) either untreated or treated with tunicamycin (10 µg·mL⁻¹) (+ tun.).
levels present in the transport null MTXrA cells and empty vector. The cells were incubated with 0.6 μM [3H]thiamine for 20 min in a transport buffer at pH 7.4 as described in Experimental procedures.

all members of the solute carrier SLC19 family including THTR1, THTR2 as well as RFC and across mammalian species (i.e. human, mouse, and hamster) (Table 1). The substitution of aspartate or histidine reverses the net charge at this position and this may strongly affect transporter function (Table 1). In order to test whether or not the conserved aspartic acid at position 93 is also essential for the transport function of the hRFC, a THTR1 and THTR2 homologue, we used site-directed mutagenesis to substitute the corresponding aspartic acid at position 88 by histidine in the hRFC. Methotrexate (i.e. an established transport substrate of RFC) transport null MTXrA murine leukemia cells were stably transfected with the vector vehicle plasmid and expression plasmids encoding for either the wild-type or the D88H mutant RFC proteins. Northern blot analysis confirmed the similar levels of RFC mRNA in the transfected cells with both the wild-type and D88H mutant RFC (data not shown). Cells were then tested for their ability to take up [3H]methotrexate. RFC transfected cells exhibited ~16-fold higher MTX transport as compared to nontransfected cells or vector transfected cells (Fig. 5). In contrast, cells expressing the D88H mutant RFC failed to exhibit MTX uptake that was above the poor background levels present in the transport null MTXrA cells and empty vector-transfected cells (Fig. 5). Based on the high conservation of TMD2 and intracellular loop 2 between the members of the SLC19 family, this D93H THTR1 mutation and D88H in the hRFC abolished transport for both thiamine and MTX, respectively, thereby, suggesting that this domain plays an important functional role in substrate binding and/or translocation.

Table 1. Sequence comparison of various members of the solute carrier family. Amino-acid alignment of the aspartate 93 in human THTR1 with various members of the SLC19 family. The conserved aspartate 93 is indicated in bold. Light shading indicates the amino acids showing conservation in all members of the solute carrier family.

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<tr>
<th>Family member</th>
<th>Δε position</th>
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<tr>
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<td>85-100</td>
<td>LPVFVLAFLDLKYKVP</td>
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Discussion

Rogers syndrome, is a rare uni-gene autosomal recessive disease, currently identified in 20 families all over the world. Rogers syndrome patients suffer from constant thiamine deficiency and display the three main disease manifestations including megaloblastic anemia, diabetes mellitus and sensorineural deafness. Some of the non-developmental manifestations in Rogers syndrome can be overcome by treatment with high doses of thiamine thus suggesting that alterations in thiamine transporter activity may play a role in the pathogenesis. Consistently, Rogers syndrome patients were found to harbor mutations in the SLC19A2, encoding for THTR1 [7–9]. Different mutations in the THTR1 (SLC19A2) gene were described in all Rogers syndrome patients [14]. There is no correlation between the type of mutation identified and the severity of disease symptoms. We therefore initiated studies aimed at the characterization of missense mutations resulting in amino-acid substitutions. Recently we have described one of the three-missense mutations that occur in Rogers syndrome families [12]. The mutation was identified in an Italian family, in which, glycine 172 is substituted by aspartate thereby resulting in an apparently misfolded THTR1 protein, that fails to undergo complete glycosylation, and is retained in the Golgi-ER compartment this resulted in the targeting of this mutant THTR1 protein to degradation [12].

We undertook the present study to determine the impact that the D93H THTR1 mutation from a French family with Rogers syndrome has on thiamine transport activity. During the early stages of our research, we were interested in selecting the appropriate Myc tag constructs (N- or C-terminal fusion) of the D93H mutant THTR1. We find here that insertion of a Myc-tag epitope at the N-terminus...
of the wild-type THTR1 retains normal expression, plasma membrane trafficking, and thiamine transport activity, whereas no detectable expression could be detected in NIH3T3 cells transfected with a THTR1 harboring a C-terminal Myc-tag. Apparently, insertion of a six-histidine tag to the N-terminal end of the wild-type THTR1 did not interfere with transporter expression and thiamine transport activity [15]. This latter result is consistent with recent studies that reported on the introduction of an N-terminal Myc-tag to the human Na+\textsuperscript{+}-glucose cotransporter [20] and the serotonin transporter [21] without interfering with their expression and transport function. The human THTR1 is shorter by 94 amino acids than its close counterpart, the hRFC, as it lacks the long C-terminus present in the latter. It is possible that the insertion of a highly charged Myc-tag (i.e. 9/17 amino acids are either negatively or positively charged) to the C-terminus in the hTHTR1 may result in protein missfolding that could be identified by the quality control mechanism in the ER, thereby leading to rapid transporter degradation. This putative degradation may gain support from the fact that the D93H-Myc mutant THTR1 (i.e. the Myc tag placed in the C-terminus) was readily translated \textit{in vitro} (Fig. 1C,D). However, one should keep in mind that not any epitope tagging at the C-terminal end of the THTR1 may necessarily result in lack of protein expression and/or rapid degradation; introduction of an enhanced green fluorescent protein (EGFP) tag at the C-terminus of THTR1 neither resulted in interference with transporter expression nor with thiamine transport activity [22]. Consistently, either hemagglutinin (HA) or green fluorescent protein (GFP) tagging at the C-terminus of the human RFC did not interfere with transporter expression, plasma membrane targeting and MTX transport activity [23]. Taken together, the tag of choice has to be carefully taken into considerations. We show here that the D93H mutant THTR1, while fused to Myc epitope tag at the N-terminal, is properly translated and targeted to the plasma membrane. Yet a previous study has shown that, upon fusion of His and Xpress tags derived from pcDNA3.1 expression plasmid (Invitrogen Inc.) with D93H mutant THTR1, this mutant protein is neither detected in the cytosolic nor in the plasma membrane fractions of transfected cells [15]. According to the topological model proposed by these authors [15], the N-terminal domain including the six His-Xpress tags and the D93H residue located in the intracellular loop between TMD2 and TMD3 are expected to come to a close proximity in the cytosolic milieu. This could result in the formation of nonphysiological ion-pairs (i.e. His-Asp) as the Xpress tag introduces as much as five Asp residues; alternatively, His 93 could augment and/or alter the metal-cation chelation capabilities of the proximal six His domain. Clearly, in both cases transporter misfolding and rapid protein degradation could be envisaged. In fact, among all mutations introduced in the His/Xpress tagged THTR1, only the D93H was undetectable [15]. Support to the notion of His tag mediated protein misfolding derives from several recent papers. First, introduction of a six His tag to lactate dehydrogenase [24] and reverse transcriptase [25] resulted in misfolding and/or loss of catalytic activity. Second, introduction of a His tag can also result in detrimental post-translational modifications; for example, His tagging of the SH3 domains of Src tyrosine kinase resulted in a spontaneous α-N-6 glucosylation which led to a severe interference with its crystallization [26].

Using transient transfections and immunofluorescent subcellular localization we find that the D93H mutant THTR1 is well expressed, undergoes an apparently complete N-linked glycosylation and is targeted to the plasma membrane. However, despite this apparently normal expression, post-translational modification (i.e. glycosylation) and trafficking to the plasma membrane, the D93H mutant transporter did not exhibit any thiamine transport activity. These results establish that the D93H THTR1 mutation disrupts thiamine uptake activity and may therefore explain the thiamine deficiency in this French family with Rogers syndrome that clinically responds only to high doses of thiamine.

We find here that the D93H mutant THTR1 as well as its D88H mutant hRFC counterpart lost thiamine and MTX transport activity, respectively. The proposed topological structure of THTR1 predicts that D93 is the first amino acid in the short intracellular loop 2 (IL2), whereas its D88 counterpart in the hRFC is embedded in the end of TMD2 [13,23]. An alignment of the deduced amino-acid sequence of the human THTR1 with that of the various members of the SLC19A family including rodent (i.e. mouse and hamster) THTR1, human and rodent THTR2, as well as RFC reveals a striking conservation at the vicinity of D93 in THTR1, THTR2 and of D88 in its counterpart, RFC. Specifically, not only is D93/D88 absolutely conserved across species in both THTR1, THTR2 and RFC, TMD2 and IL2 are extremely well conserved (Table 1). Consistently, we recently identified the very same D88H mutation (along with additional mutations in TMD2) in GW1843-resistant leukemia GW70/LF cells that displayed a markedly altered transport of antifolates and folates [27]. Moreover, recent studies demonstrate that elimination of the negative charge at the D88 of the hRFC (e.g. D88V) results in a complete loss of its MTX transport activity [16]. It was further found that this negatively charged residue (i.e. D88) is absolutely essential for folate (i.e. leucovorin) and antifolate (i.e. MTX) transport as it presumably forms an ion-pair with a positively charged residue (i.e. R133) in TMD4 of the hRFC. It was therefore concluded that this charge-pair presumably allows for a proper tertiary structure to occur that is absolutely essential for folate and antifolate transport. Hence, it is possible that the conserved D93 residue in THTR1 also plays an important role in the formation of a certain tertiary structure that is crucial for thiamine binding and/or substrate translocation.

Multiple amino-acid substitutions are known to disrupt RFC transport function [28]. As the number of patients carrying THTR1 missense mutations is rather limited due to the fact that Rogers syndrome is a relatively rare disorder [14], it would be interesting to test whether other substitutions in conserved residues of the orthologue hRFC can inactivate the thiamine transport function of THTR1 and THTR2 as well. Therefore, further comparative mutagenesis studies between the three members of the solute carrier family should provide useful information regarding the specificity and functionality of these transporters.
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References