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Structure–function analysis of the highly conserved charged residues of the membrane protein FT1, the main folic acid transporter of the protozoan parasite *Leishmania*

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ABSTRACT

The main plasma membrane folate transporter FT1 of *Leishmania* belongs to the novel FBT family which is part of the major facilitator superfamily. We have investigated the role of the 10 most conserved charged amino acids of FBTs by site directed mutagenesis. The functions of the mutated proteins were tested for their capacity to transport FA, to sensitize methotrexate resistant cells to methotrexate, for protein production, and for protein localisation. Of the 10 conserved charged amino acids that were mutated to neutral amino acids, all had effects on FT1 transport activities. Only four of the 10 initial mutants (K116L, K133L, R497L, and D529V) retained between 15% and 50% of FT1 activity. The R497 residue was shown to be involved in substrate binding. When the charged conserved residues at position 124, 134, 179, 514, 537 and 565 were changed to neutral amino acids, this led to inactive proteins but the generation of new mutants D124E, R134K, D514E and D537E regained between 20% and 50% of wild-type FT1 activity suggesting that the charge is important for protein function. The mutated protein D179E had, under our standard experimental conditions, no activity, while E565D was completely inactive. The differential activity of the mutated proteins was due either to changes in the apparent K_m or V_{max}. Mutagenesis experiments have revealed that charged amino acids were essential for FT1 stability or activity and led to a plausible model for the transport of folic acid through FT1.

1. Introduction

Folic acid (FA) and related derivatives are conjugated pterins that, once reduced, are essential co-factors in one carbon transfer metabolism including the synthesis of thymidine, methionine and depending on the organisms of purines, serine, glycine and other molecules (reviewed in [1]). Some organisms are capable of de novo synthesis of FA (and of pterins) while others need to get these molecules from the environment. Membrane transport of FA is thus essential for several organisms and a diversity of membrane proteins are involved in the transport of FA derivatives. For example, the GPI-anchored membrane folate receptors mediate FA uptake by endocytosis and a number of facilitative organic anion carriers, including the reduced folate carrier, transport FA in mammals (reviewed in [2]). Recently, a proton coupled low pH folate transporter was isolated [3] and membrane transporters part of the ATP Binding Cassettes (ABC) family were shown to efflux FA derivatives (reviewed in [4]). Mammals [5] and plants [6] have mitochondrial carrier family proteins for the transport of FA into mitochondria. All these proteins are structurally different but have in common the transport of folate derivatives.

A further novel class of transporters, the Folate Biopterin Transporters (FBT) (www.tcdb.org/index.php) were recently described. They were first identified in the protozoan parasite *Leishmania* and were shown to transport biopterin [7,8] or FA [9,10]. Other members of the FBT family are likely to transport other substrates [11]. FBT homologues were found in the parasites responsible for sleeping sickness or Chagas diseases [11], malaria [12] or toxoplasmosis [13]. FBT homologues in cyanobacteria or in the plant Arabidopsis were also shown to transport FA and a number of derivatives [14]. The FA analogue methotrexate (MTX) is a widely used drug and the activity/mutations of some members of this diverse class of transporters can influence MTX accumulation and resistance to MTX. Due to the strategic importance of FA in one carbon transfer reaction a number of distinct membrane proteins have thus evolved in various organisms to transport FA derivatives.

The protozoan parasite *Leishmania* is distributed worldwide and is responsible for considerable morbidity and mortality [15,16]. Few drugs are available against this parasite but their FA metabolism is sufficiently different from their hosts that

Abbreviations: FA, folic acid; FBT, Folate Biopterin Transporter; MTX, methotrexate; NHS, *N*-hydroxysuccinimide; TMS, trans membrane segments.

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specific inhibitors of this pathway may eventually be found [1,17]. Indeed, several efforts are devoted to the generation of antifolates against *Leishmania* [18–21].

Leishmania is auxotroph for both pterins and folates and not surprisingly transport activities were described for these molecules in *Leishmania* [22,23]. The FA transport was decreased in MTX resistant cells [24–26]. The biopterin transporter BT1 was first isolated by functional cloning [7] and it was soon realised that this transporter is part of a family of proteins distantly related to the major facilitators. This family was named FBT (www.tcdb.org/index.php) and in addition to BT1, two more members were functionally characterized. FT5 corresponds to a high affinity/low capacity folate transporter [9] and FT1 to the main folate transporter of *Leishmania* [10]. The genome sequence of *Leishmania* has revealed 14 members of the FBT family with a varying degree of conservation but with several amino acids conserved not only between the *Leishmania* sequences but also in the FBT proteins found in other parasites, bacteria and plants. We used FT1 as the paradigm for studying FBT structure function relationship and have concentrated on conserved charged amino acids. The studies reported here are consistent with the importance of charged amino acids in the function of membrane proteins.

2. Material and methods

2.1. Parasites and culture

Leishmania tarentolae strain TarII MTX-1000.6 was described previously and has no measurable folic acid or methotrexate transport [26]. *L. tarentolae* promastigotes were transfected by electroporation as reported previously [27]. Growth inhibition was determined at various concentrations of MTX as described using $OD_{600\ nm}$ [28].

2.2. Western blot analysis

Total *Leishmania* proteins (30 μg) were run on 12% polyacrylamide gels and blotted onto nitrocellulose membranes as described [29]. The blots were blocked overnight in 5% skimmed milk in PBS. A monoclonal anti-α-tubulin antibody (Sigma, Oakville, ON, Canada) directed against a conserved amino-terminal peptide of the bovine α-tubulin or an antibody against the Green Fluorescent Protein (GFP) (Invitrogen, Burlington, ON, Canada) were diluted 1:3000 in PBS containing 0.1% Tween 20 (PBS–Tween) and incubated for 1 h with the blot. The blot was washed 3 × 5 min in PBS–Tween and incubated with horseradish peroxidase-conjugated sheep-anti mouse IgG for α-tubulin and sheep-anti rabbit IgG for GFP (Amersham Biosciences, Baie D'Urfé, QC, Canada) diluted 1:10,000 in PBS–Tween. Reactions were revealed with the ECL Plus chemiluminescent substrate (Amersham Biosciences, Baie D'Urfé, QC, Canada), and autoradiography.

2.3. Site directed mutagenesis

Mutagenesis was performed using the quick change site-directed mutagenesis kit (Stratagene, Mississauga, ON, Canada). Mutagenic primers were designed to incorporate the desired mutations in FT1. Mutations were inserted within the *FT1* open reading frame fused with GFP at its C-terminus. After sequencing of the whole open reading frame, mutated *FT1GFP* were subcloned into the *Leishmania* expression vector pSPαHYGα [30]. A total of 19 point mutations were generated and are listed in Table 1.

2.4. Confocal microscopy

Parasites were washed in PBS, and resuspended at a density of 10^7 cells/ml. Cells were immobilized in a 0.5% low melting agarose PBS buffer and mounted on microscope slides with coverslips. Samples were viewed with an Olympus FV300 confocal scanning laser system installed on an Olympus IX-70 inverted microscope with an argon laser. Visualization of the fluorophore was achieved using a 488 nm excitation filter and 510/530 nm emission filter. Samples were scanned for green fluorescence using a 100× objective (numerical aperture, 1.60). Images were obtained using the Olympus Fluoview 300 software.

Table 1
Activities of mutated versions of FT1.

Amino acid	Conservation[a] (%)	MTX susceptibility (μM)[b]	Protein production[c]	Folate uptake (pmol/10^8 cells/min)[d]
WT	–	–	–	0.22 ± 0.05
MTX1000.6	–	>1000	–	0.02 ± 0.01
FT1GFP-WT	–	<50	+	1.44 ± 0.17
-K116L	89	<50	+	0.63 ± 0.15
-D124V	100	>1000	+	0.03 ± 0.02
-D124E	100	420	+	0.19 ± 0.06
-K133L	57	<50	+	0.64 ± 0.13
-R134L	89	>1000	+	0.03 ± 0.02
-R134K	89	<50	+	0.59 ± 0.08
-D179V	89	>1000	+	0.05 ± 0.04
-D179E	89	350	+	0.03 ± 0.03
-E270L	55	<50	+	1.14 ± 0.17
-R497L	92	<50	+	0.65 ± 0.29
-D514V	78	>1000	–	0.01 ± 0.01
-D514E	78	<50	+	0.29 ± 0.08
-D514N	78	>1000	+	0.01 ± 0.01
-D529V	92	<50	+	0,25 ± 0,05
-D529E	92	<50	+	0.45 ± 0.02
-D537V	81	>1000	+	0.04 ± 0.02
-D537E	81	<50	+	0.86 ± 0.01
-E565L	100	>1000	+	0.02 ± 0.01
-E565D	100	>1000	+	0.02 ± 0.01

[a] % Conservation at the same position among the 37 proteins of the alignment.
[b] MTX susceptibility of *L. tarentolae* MTX 1000.6 strain expressing varying versions of FT1. Average of triplicate measurements.
[c] Detection of the production of FT1GFP proteins by Western blot and their localisation within the plasma membrane.
[d] Folate transport after 10 min. Average of triplicate measurements.

2.5. Immunofluoresence

For live cells, parasite were washed twice in PBS, and resuspended in 50 μl of suspension buffer (PBS, 1% glucose and 10% fetal calf serum) at 10^7 cells. Cells were incubated on ice for 1 h with occasional mixing with 50 μl of PBS containing an anti-GFP labelled with the Alexa Fluor® 647 dye (Invitrogen, Burlington, ON, Canada) diluted 1:100. Cells were washed twice with ice cold PBS and resuspended in 250 μl of ice cold PBS. Cells were fixed on Lab-TekII chambered cover glass slides (Fisher, Ottawa, ON, Canada) coated with poly-L-lysine, overlaid with PBS and processed for imaging as described above.

For permeabilized cells, parasites were washed twice in PBS, and resuspended at 10^7 cells in 500 μl of PBS contaning 4% paraformaldehyde during 30 min at room temperature. After incubation and washes in PBS, the cells were fixed on Lab-TekII chambered cover glass slides coated with poly-L-lysine. Attached cells were washed twice with PBS and permeabilized with 500 μl of PBS containing 0.1% of Triton-X-100 during 30 min at room temperature. The fixed permeabilized cells were washed in PBS and incubated 1 h with 250 μl of PBS containing an anti-GFP Alexa Fluor® 647 dye-labeled (Invitrogen, Burlington, ON, Canada) diluted 1:100. After this final incubation, the cells were washed twice with PBS and overlaid with PBS and processed for imaging as described.

2.6. Folate and methotrexate transport assays

L. tarentolae MTX 1000.6 expressing the various FT1 versions were harvested during the mid-log phase. 1×10^8 cells were washed and resuspended in folate deficient medium fdDMEL with 115 nM of [^{3}H] folate (43.2 CI/mmol) or [^{3}H] methotrexate (12 CI/mmol) (Moravek Biochemicals, Brea, CA, USA). Accumulation was measured as previously described [26]. Folate uptake was normalized with cells numbers and the background transport was removed by subtracting the accumulation value obtained on ice. For the MTX inhibition transport assay with *N*-hydroxysuccinimide (NHS)-MTX, the cells were pre-treated with NHS-MTX (416 μM) during 20 min and washed three times with fdDMEL prior to transport studies with 416 nM of [^{3}H] methotrexate (12 CI/mmol).

2.7. Kinetics analysis

The transport kinetic parameters V_{max} and apparent K_m for folic acid were measured while using seven different folic acid concentrations (10–1000 nM); during the linear phase of accumulation (2 min). Kinetic parameters have been determined by linear regression analysis and Michaelis–Menten analysis (Sigma Plot).

2.8. Affinity labelling of FT1 with NHS-[^{3}H]MTX

The preparation of unlabeled and radiolabeled NHS-MTX was performed as described by others [31]. Cells (8×10^8) expressing either the wild type or mutated versions of FT1 were exposed during 20 min to NHS-[^{3}H]MTX at a final concentration of 83 μM in fdDMEL. After three washes with fdDMEL, proteins were extracted and their concentrations determined. Electrophoretic separation of the proteins (100 μg) was performed in 10% polyacrylamide gel with SDS. 5 mm gel fractions were sliced and resuspended in 1 ml of soluene-350 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) overnight at room temperature. Scintillation liquid was added and radioactivity was detected in a scintillation counter.

3. Results

3.1. Conservation of amino acids in the FBT

The FBT proteins are part of a novel family of membrane proteins belonging to the major facilitator superfamilly that are found in *Leishmania* and related trypanosomatid parasites, in the Apicomplexa parasites such as *Plasmodium* and *Toxoplasma*, in plants, and in cyanobacteria (Supplementary Fig. S1) [14]. Few of these proteins have been functionally characterized but FT1 was shown to be the main folate transporter in the parasite *Leishmania* [10]. A clustalW alignment of thirty seven hypothetical proteins from *Leishmania* (13), *Trypanosoma* (9), *Plasmodium* (2), *Cyanobacteria* (6), and *Arabidopsis thaliana* (7) all belonging to the FBT family, has highlighted conserved amino acids (Supplementary Fig. S1). As deduced from phylogenetic analysis [11] the highest degree of identities of FT1 was with the FBT proteins of *Leishmania* and *Trypanosoma*. The trypanosomatid proteins were generally larger with a ~120 amino acids insertion in the middle of the protein located between the putative trans membrane segments (TMS) 6 and 7 (Fig. 1A). Sequence alignment has permitted to pinpoint several highly conserved amino acids with even some of them completely invariant among the 37 proteins (taking the FT1 as a paradigm this would include D124, Y136, G220, G563 and E565). Two of these invariant residues are charged and these type of residues often play either a structural or functional role in membrane proteins [32–34]. Recently, a charged residue was found to be important in FA binding in the mammalian reduced folate carrier [31]. This level of conservation prompted us to carry out a structure–function analysis of conserved charged amino acids in the activity of FT1. We first targeted the 10 most conserved (at least 80% conservation) charged amino acids within the FBTs (Table 1). K133 was selected despite it was found in only 57% of the 37% proteins because the remaining 43% proteins had an arginine residue at this position suggesting that the positive charge may be important. Position E270 is not part of the 10 selected conserved amino acids and is not highly conserved within the FBTs outside *Leishmania* and was chosen as a mutagenesis control.

The FBT family was recently characterized and no experimentally validated topological model is available. A number of software was used to try to determine the most likely topological model for FT1. The favoured model predicted 14 TMSs for FT1 with the N- and C-terminus inside the cell (Fig. 1A). This model was predicted by both the Prodiv_TMHMM_0.91 [35] and the Ensemble 1.0 software [36]. BT1, also transporting folic acid and biopterin, was also predicted to contain 14 TMSs (result not shown). FT1 was fused to the Green Fluorescent Protein (GFP) at its C-terminus and was found to be functional (see below) and to localise to the plasma membrane (Fig. 1B). Non-permeabilized cells did not react with an Alexa-labelled anti-GFP antibody but Triton-X-100 permeabilized cells were shown to react with this antibody (Fig. 1B) suggesting that indeed the C-terminus of FT1 was intracellular. An N-terminal GFP-FT1 fusion was non functional (results not shown). The software TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) and SOSUI [37] predicted FT1 with 12 TMSs but with the N- and C-terminus outside and thus this topology was not retained as it was not in line with the results shown in Fig. 1B. The TMHMM 2.0 software [38] also forecasted the C-terminus of FT1 to be intracellular but in this case FT1 had an unusual number of 11 predicted TMSs (not shown). While we cannot exclude that this may be correct, FT1 is a member of the major facilitators which usually are predicted to contain 12 or 14 TMSs [39]. A putative topological model for FT1 of 14 TMS is shown in Fig. 1 and the localisations of the mutated amino acids are indicated. While hypothetical, this model indicates that amino acids may be part of intracellular loops or close to the plasma

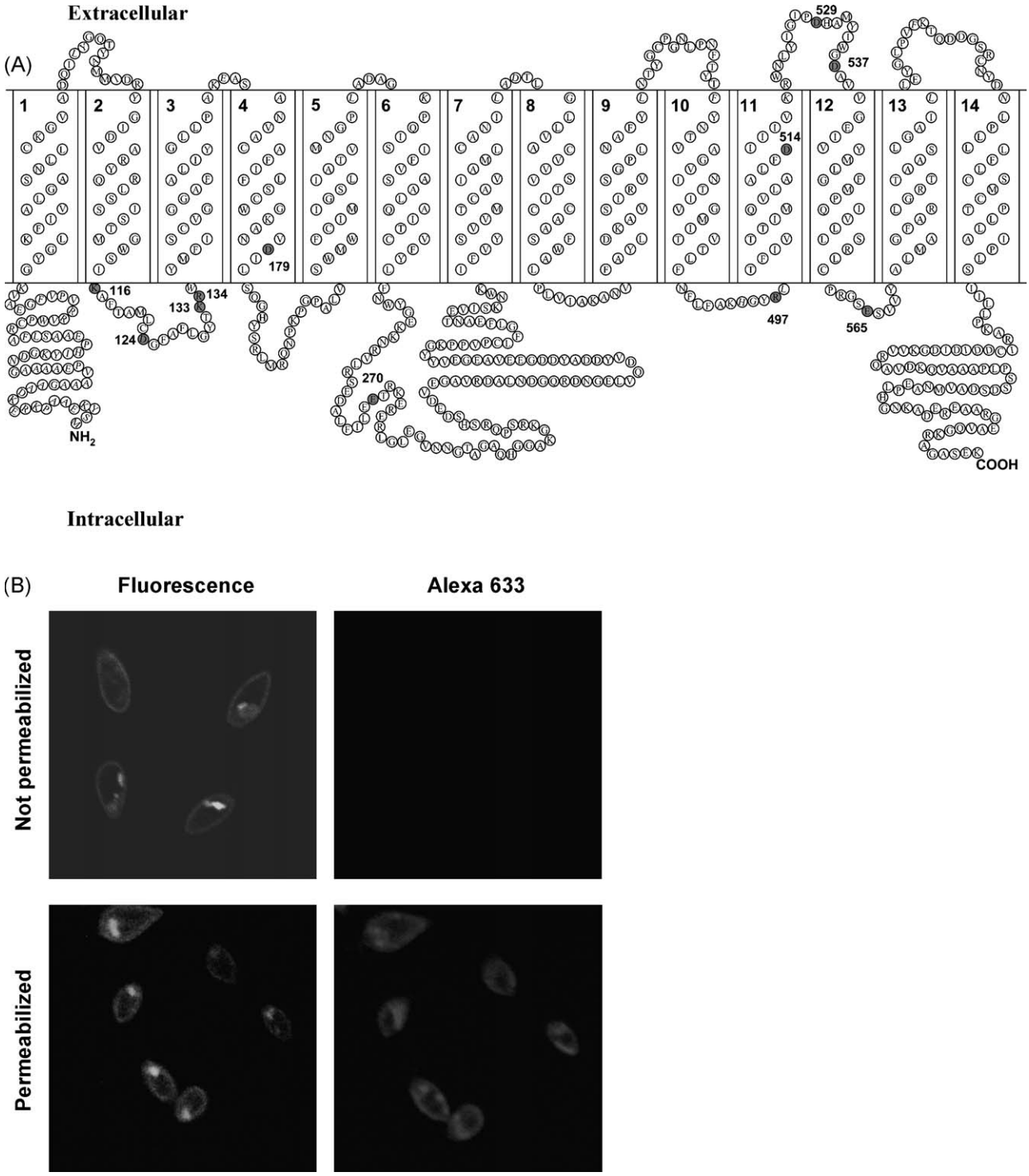

Fig. 1. FT1 putative topology. (A) The topology for FT1 was determined using a number of software and the preferred structure determined from the Provid_TMHMM_0.91 program is shown. This algorithm predicted a 14 TMS protein with the N-terminus and the C-terminus inside the cell. Every single amino acid is shown and conserved charged amino acids that were mutated in this study were highlighted. (B) Intracellular localisation of the C-terminus of FT1. Cells were transfected with a functional FT1-GFP C-terminal fusion and were located by fluorescence (left panel) or by reacting with anti-GFP antibody (right panels) in either intact or Triton-X-100 permeabilized cells.

membrane (position 116, 124, 133, 134, 497, 565), in putative extracellular loops (position 529, 537), or finally within predicted TMSs (position 179, 514).

3.2. Site directed mutagenesis

All charged conserved residues were mutated by site directed mutagenesis. To try to minimize size effects, K, E and R residues were replaced by L while D were replaced by V. In all cases, the mutated genes were sequenced to ascertain the presence of only the desired mutation. The functions of the mutated proteins were tested for their capacity to transport FA, to sensitize MTX resistant cells to MTX, for protein production, and for protein localisation. When the mutant proteins were inactive, a second round of mutagenesis was done in order to change the amino acid but to keep the charge. These mutants were analysed similarly. Several

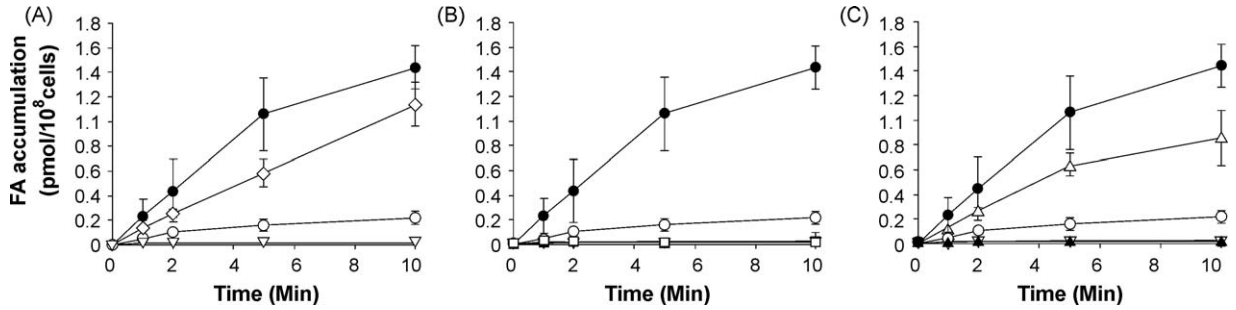

Fig. 2. Folic acid accumulation by FT1 and its mutated versions. Accumulation of folic acid was measured with time in *L. tarentolae* MTX1000.6 cells (▽), in *L. tarentolae* wild-type cells (○) and in *L. tarentolae* MTX1000.6 cells transfected with FT1GFP (●); or with the different mutated constructs. A selection of these is shown. (A) Mutations of conserved charged residues and retention of (part of) FT1 activity; (B) Mutations of conserved charged residues and loss of FT1 activity; (C) Mutations of conserved charged residues to neutral amino acids and loss of FT1 activity but rescuing by mutations to charged amino acids. FT1GFP-E270L (◇); FT1GFP-E565L (□); FT1GFP-E565D (■); FT1GFP-D537V (▲); FT1GFP-D537E (△). Average of triplicate measures.

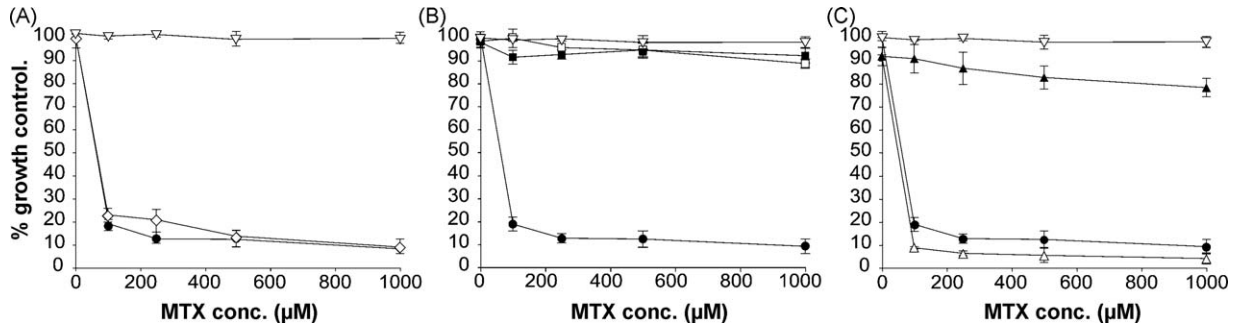

Fig. 3. Methotrexate susceptibility of *Leishmania* cells. Susceptibility of *Leishmania* MTX1000.6 transfected with various versions of FT1 to MTX was measured as indicated under experimental procedures. (A) Mutation of conserved charged residues to neutral amino acids leading to proteins conferring susceptibility to MTX. Mutations of conserved charged amino acids to neutral amino acids leading to proteins not conferring susceptibility to MTX but when changed to charged amino acids conferred no (B) or full susceptibility (C) to MTX. *L. tarentolae* MTX1000.6 cells (▽); *L. tarentolae* MTX1000.6 cells transfected with FT1GFP (●); FT1GFP-E270L (◇); FT1GFP-E565L (□); FT1GFP-E565D (■); FT1GFP-D537V (▲); FT1GFP-D537E (△). Average of at least triplicate measurments.

phenotypes were observed. In some instances the conserved charged amino acids was only moderately important. In other mutants the charge of the amino acid appeared important and some of the conserved charged amino acids were essential for the folate transport mediated by FT1. The results of the mutagenesis are summarised in Table 1.

3.3. Mutations of conserved charged amino acids and retention of (part of) FT1 activity

The *L. tarentolae* MTX1000.6 mutant is highly resistant to MTX and under standard experimental conditions neither MTX nor FA is accumulated within these resistant cells [9,10,26]. Transfection of these cells with a *Leishmania* expression vector containing the *FT1* gene fused at its C-terminus with GFP (FT1GFP-WT) now led to FA transport in the *L. tarentolae* MTX1000.6 background (Fig. 2) and these cells became sensitive to MTX (Fig. 3). The FA accumulation was higher than what observed in wild-type cells (Fig. 2, Table 1). The drug sensitization was due to the re-acquired capacity of *L. tarentolae* MTX1000.6-FT1GFP-WT to transport MTX (Fig. 4). As a first mutagenesis test, we mutated E270L, an amino acid that is not highly conserved among the FBT outside the *Leishmania* proteins (Supplementary Fig. S1, Table 1). *L. tarentolae* MTX1000.6 cells transfected with the FT1GFP-E270L construct were shown to transport FA similarly to cells transfected with FT1GFP-WT (Fig. 2A) and also rendered cells sensitive to MTX (Table 1). As expected from this phenotype, the FT1GFP-E270L protein was produced and located to the plasma membrane of the parasite (results not shown).

Of the 10 conserved charged amino acids that were mutated to neutral amino acids, all had effects on FT1 transport activities. These transport activities were compared to cells transfected with FT1GFP-WT and varied from a decrease of ~50% to no activity at all (Table 1). Only four of the 10 initial mutants retained some FT1 activity when charged conserved residues were changed to neutral amino acids. For three mutations (K116L, K133L, and R497L) we observed a 50% decrease in the kinetics of folic acid uptake (Table 1). While the capacity to transport folic acid was altered, cells producing these proteins were as susceptible to MTX as cells transfected with the FT1GFP-WT construct (Table 1). The three mutated proteins were produced and localised to the plasma membrane (results not shown). The FT1GFP-D529V construct still exhibited some transport activity but this was only ~15% of the overexpressed FT1GFP-WT activity (Table 1). Western blot

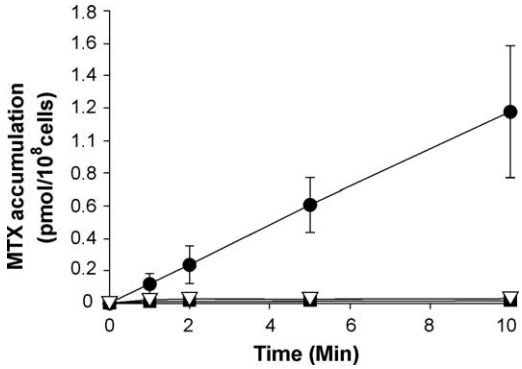

Fig. 4. Methotrexate accumulation by FT1 and selected mutated versions. Accumulation of MTX was measured with time in *L. tarentolae* MTX 1000.6 cells (▽);*L. tarentolae* MTX1000.6 cells transfected with FT1GFP (●) or FT1GFP-E565D (■). Average of triplicate experiments.

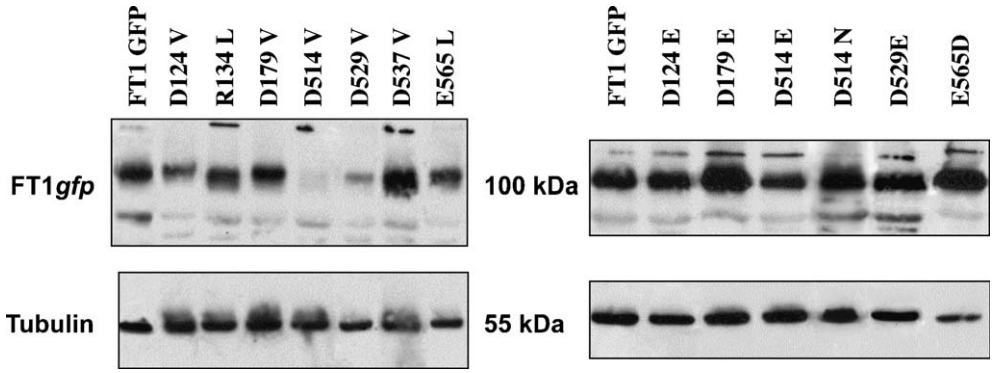

Fig. 5. Expression of FT1 mutated version in *L. tarentolae* MTX1000.6. Total proteins were extracted from *L. tarentolae* MTX1000.6 transfectants, 30 μg were run of PAGE gels, transferred to nitrocellulose and reacted with an anti-GFP antibody (Invitrogen). The reaction was revealed with an horseradish peroxidase-conjugated sheep–anti rabbit IgG. The α-tubulin protein was also detected by western blot analysis to monitor the amount of protein layered in the gel.

analyses have revealed that the FT1GFP-D529V protein was notably less produced than other mutants (Fig. 5), hence possibly explaining the reduced activity of this mutated protein. Nonetheless, sensitization to MTX was observed in this mutant.

3.4. Mutations of conserved charged amino acids and loss of FT1 activity

When the charged conserved residues at position 124, 134, 179, 514, 537 and 565 were changed to L or V, this led to FT1GFP proteins that could not transport FA (Table 1, Fig. 2B). Two of these amino acids, D124 and E565, are conserved in all FBT proteins (Supplementary Fig. S1, Table 1). Transfectants with all of these mutated constructs were still resistant to MTX (Table 1, Fig. 3B and C) and it was found that it was due to an inability to transport MTX (Fig. 4 and results not shown). Despite being inactive, all FTGFP mutated proteins were expressed as determined by Western blot analyses with the exception of the FT1GFP-D514V version (Fig. 5). The expressed mutated proteins were also found to localise to the plasma membrane as determined by confocal microscopy (Fig. 6) showing that the absence of FA transport activity in D124V, R134L, D179V, D537V and E565L was not due to gross defect in protein production or localisation. FT1 is known to be regulated with the growth stage being degraded in stationary

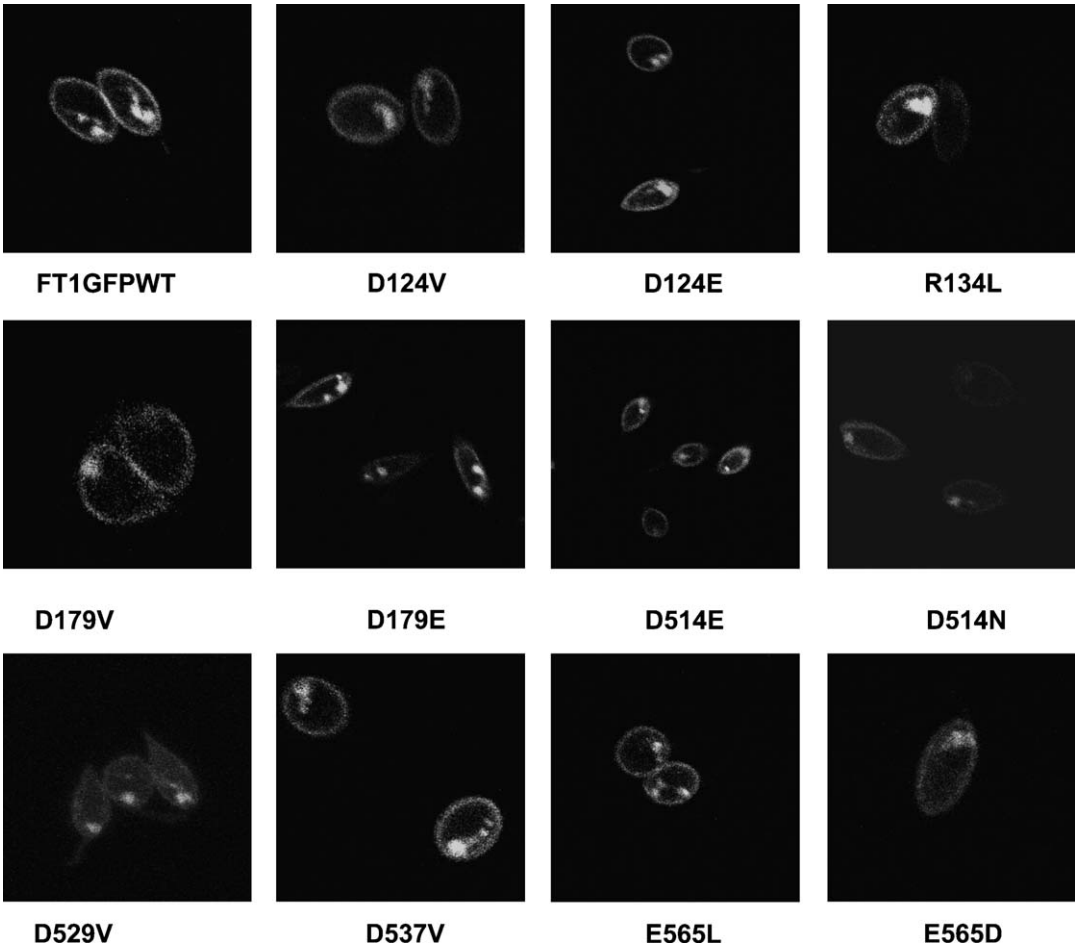

Fig. 6. Subcellular localisation of FT1-GFP proteins. Confocal scanning microscopy of FT1GFP versions present in *L. tarentolae* MTX1000.6.

phase [10] and all mutated proteins were similarly regulated (results not shown).

3.5. The role of the charge in keeping FT1 activity

Since we targeted charged amino acids, we investigated whether a mutation keeping the charge intact would lead to a functional FT1 protein. We targeted the six residues where a mutation led to an inactive protein. In addition, we included the D529E version as the D259V mutant had a reduced FA accumulation and less protein produced (Table 1). Transfectants expressing the FT1GFP-D529E were found to transport more FA than the D529V version but still it was only ~30% of the level of transfectants expressing FT1GFP. Contrarily to the D529V version, the D529E version of FT1 was well expressed (Fig. 5) and localised to the membrane (result not shown). *L. tarentolae* MTX1000.6 cells transfected with R134K or D537E were capable of accumulating FA at about 50–70% of the activity of cells transfected with FT1GFP (Table 1, Fig. 2C) and became sensitive to MTX at a level similar to cells transfected with FT1GFP (Fig. 3C). These active proteins were produced and localised to the plasma membrane (results not shown). Transfection of FT1GFP-D124E and D514E versions restored some FA transport (~15–30%) (Table 1). Concomitant to this small gain in activity we observed a partial (D124E) or total (D514E) sensitization to MTX (Table 1). While the FT1GFP-D514V protein was not produced, the D514E version was produced (Fig. 5) and localised to the plasma membrane (Fig. 6). Finally, the FT1GFP-D179E and FT1GFP-E565D mutant proteins were produced (Fig. 5) and localised to the plasma membrane (Fig. 6). The FT1GFP-E565D could neither transport FA (Fig. 2B) nor could it sensitize *L. tarentolae* MTX1000.6 for MTX (Fig. 3B). This was due to an inability to transport MTX (Fig. 4). FT1GFP-D179E could not transport FA under our standard assay conditions but could partly sensitize cells to MTX (Table 1). This was due to the high MTX concentration tested, because when we increased the concentration of FA in our transport assay (from 115 nM to 1 μM) we then observed that cells transfected with FT1GFPD179E could transport FA (result not shown).

3.6. Kinetics parameters of mutated FT1 proteins

All the mutated FT1 proteins had an altered ability to accumulate FA (Table 1). To look at the possible basis for this changed in activity, we calculated the V_{max} and apparent K_m of the FT1 versions that still exhibited measurable FA transport activities. Mutations at position 116 and 133 did not alter significantly the apparent K_m but did alter slightly the V_{max} of the proteins (Table 2). Mutations at positions 134, 497, 529 and 537 increased the apparent K_m by ~6-fold but with minimal changes in V_{max} except for the FT1 D529V version where both the K_m and V_{max} were significantly changed (Table 2). Interestingly cells containing a FT1D529E version also had a high K_m but the negative charge at position 529 brought back the protein with the V_{max} of a wild-type protein (Table 2).

Table 2
Kinetic parameters of FT proteins.

Transfectants	K_m (nM)	V_{max} (pmol/10^8 cells/min)
FT1GFP-WT	53 ± 18	0.38 ± 0.13
FT1GFP-K116L	25 ± 9	0.08 ± 0.02
FT1GFP-K133L	40 ± 14	0.11 ± 0.06
FT1GFP-R134K	344 ± 153	0.22 ± 0.06
FT1GFP-R497L	315 ± 164	0.33 ± 0.17
FT1GFP-D537E	400 ± 66	0.38 ± 0.06
FT1GFP-D529V	442 ± 113	0.07 ± 0.08
FT1GFP-D529E	473 ± 172	0.36 ± 0.18

Average of triplicate measurements.

3.7. A charged amino acid involved in the binding of folate

The cross-linking reagent NHS-MTX is a useful tool for investigating the interactions of the folate transporters and their substrate and has been used as an affinity inhibitor [31]. We synthesized NHS-MTX which was shown to inhibit the transport of MTX (Fig. 7A) by binding to FT1 (Fig. 7B). Indeed, more than 75% of FT1 transport activity was inhibited if cells were pre-incubated with NHS-MTX prior to transport studies and a 100 kDa band (corresponding to FT1-GFP, see Fig. 5) was shown to bind NHS-[^{3}H]MTX (Fig. 7B). Mutations in FT1 changing its transport properties may be due to decreased binding to its substrate. If this were the case, NH-MTX would be predicted to be less inhibitory to mutated proteins. We studied the inhibitory effect of NHS-MTX on cells having mutated FT1 still capable of some transport activity. The transport of MTX in proteins containing FT1E270L was, as expected (this was a control mutation with no phenotype (Table 1), inhibited by NHS-MTX (Fig. 7A). We investigated the transport of MTX in cells having either FT1K116L or FT1R497L which have either defect in their V_{max} of K_m, respectively (Table 2). While marked inhibition of transport was observed for K116L after pre-incubation with NHS-MTX this was not the case for FT1R497L. To prove that this lack of inhibition was due to a decrease binding, we used NHS-[^{3}H]MTX and showed that while the wild-type protein indeed binds this substrate, this

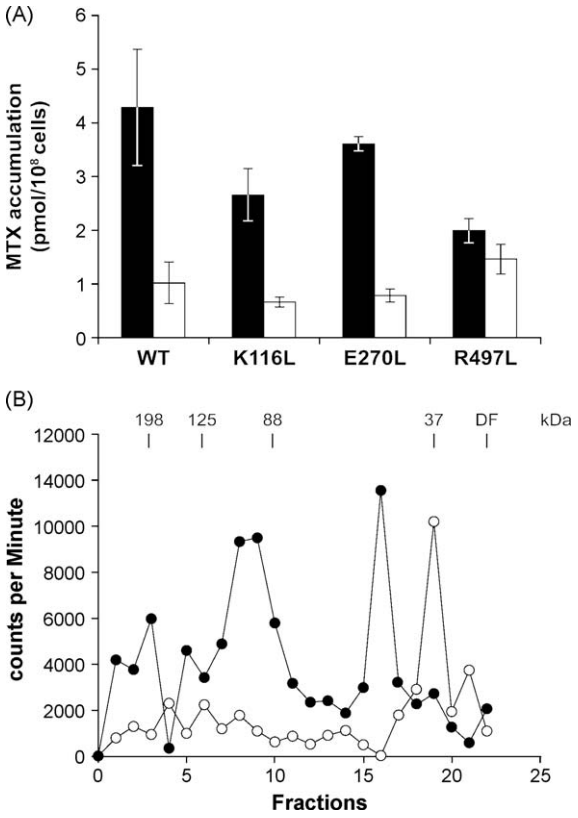

Fig. 7. *N*-hydroxysuccinimide methotrexate (NHS-MTX) inhibition and binding to the Leishmania FT1 protein. (A) Transport inhibition of FT1 expressed in *L. tarentolae* MTX1000.6. Cells were treated with NHS-MTX (416 μM) during 20 min, washed, prior to MTX transport studies over 10 min. MTX was compared between NHS-MTX treated (white bars) and untreated cells (grey bars) expressing wild-type and mutated FT1 proteins. Average of three independent experiments. (B) Affinity radiolabeling of FT1 proteins with NHS-[^{3}H]MTX. Cells were incubated with NHS-[^{3}H]MTX as described under Section 2. Total proteins were separated with 10% polyacrylamide gels containing SDS and 5 mm slices were cut and their associated radioactivity were counted and plotted according to a molecular weight standard. This experiment was repeated twice with essentially the same profile on each occasion. FT1 wild-type protein (●), FT1R497L (○).

interaction is lost in R497L as evidenced by the absence of radioactivity in the 100 kDa fraction (Fig. 7B).

4. Discussion

Several types of membrane proteins are able to transport FA derivatives. Recently, a novel class of proteins part of the FBT family was shown also to transport either biopterin or FA and members were found in cyanobacteria, protozoa and plants, [7–10,14]. These proteins, and the molecule they transport are important for cell physiology and host-pathogen interactions [40,41] and a better understanding of these proteins is warranted. An alignment of several members of the FBT proteins found in phylogenetically distinct organisms has revealed several highly conserved amino acids, some of which could be important for function of FBT members. We used FT1 as a paradigm for studying structure–function of this newly described class of proteins. Several of the highly conserved amino acids were charged and due to the known role of these residues in membrane proteins, including proteins of *Leishmania* [33,42], we mutated the 10 most conserved charged residues of FT1.

No topological model for any FBT protein has yet been experimentally tested. Computer algorithms have depicted the *Synechocystis* slr0642 protein with 12 TMS [14] but all algorithms that we have used depicted FT1 with either 14 or 11 TMSs when taking into account that the C-terminus of the protein needed to be intracellular, as we have experimentally verified (Fig. 1B). Tagging technology should be useful to further validate the proposed topology but a GFP-FT1 N-terminal fusion was non functional (not expressed) suggesting that smaller tags may be required. However, an N-terminal fusion with another related FBT protein suggested that the N-terminus of the latter is indeed located in the cytosol (L.D. and M.O., unpublished observation), further supporting a 14 TMS topology.

Position E270 is conserved only within the kinetoplastidae (Supplementary Fig. S1) and thus it was a useful control to show that mutation of this residue to E270L did not affect significantly FT1 function (Figs. 2A and 3A, Table 1). Mutations of the highly conserved K116, K133 and R497 residues had only a partial effect on FT1 function (Table 1). Mutations at position K116 and K133 decreased the V_{max}, while the K_m of the R497L mutant was increased (Table 2). Thus, despite a similar fold decrease in the activity of FT1 in these mutants, the mechanisms by which this is achieved differed. The exact amino acid corresponding to position 133 in FT1 is either an arginine or a lysine in all FBTs (Supplementary Fig. S1). It would appear, however, that this positive charge is not essential for FBT activity (Table 1). According to our topological model, the three positively charged residues K116, K133 and R497 are located close to the plasma membrane (Fig. 1A) and it is possible that they may interact with the negatively charged head groups of the membrane phospholipids [43] or to the ionized carboxyl group of folates. The change in K_m in R497L (Table 2) is suggestive of direct interactions between the substrate and the charged residue at this position. This was corroborated by a lack of inhibition of MTX transport by NHS-MTX (Fig. 7A) due to an absence of binding to cells expressing FT1R497L (Fig. 7B). In our topological model, R497 is localised into the intracellular loop between TMD 10 and 11 of FT1. This amino acid could be involved in the translocation pathway of FA from the channel of the protein to the cytoplasm. This interaction however, is not essential for transport (Table 2) suggesting that other amino acids in FT1 can compensate for the lack of a positive charge at position 497.

Mutation of another conserved charged residue (D529V) also left some activity to FT1 but this mutated protein transported less FA than proteins mutated at positions 116, 133 and 497. However, FT1 D529V was less stable or less expressed and its localisation at the level of the plasma membrane was less clear (Fig. 6). This explained also why the V_{max} of FA transport in these transfected cells was decreased (Table 2). Changing D529V to D529E increased the amount of protein produced (Fig. 5) and its V_{max} (Table 2) but still the FA transport activity was notably less than for the other mutants. These results suggested that position 529 may be important for substrate recognition and replacing the aspartic acid changed the K_m.

For four other interrogated positions, the charge of the amino acid seemed to be essential. Indeed, when the conserved charge of position 124, 134, 514 or 537 were changed to neutral amino acids, FT1 became inactive (Table 1, Figs. 2 and 3). However when the charge was retained (e.g. changing D to E or R to K) these mutated proteins kept at least part of their FA transport activity. The FT1D514V protein was not expressed at all (Fig. 5) but since the FT1D514E version was expressed, it suggested that the negative charge at this position is important for either protein stability and/or activity. To further test the role of position 514, we generated an additional mutant, FT1D514N. This mutated protein was produced (Fig. 5) and expressed at the plasma membrane (Fig. 6) but it remained inactive (Table 1). Thus the charge at position 514 is essential for activity but other factors, possibly the size of the amino acid side chain, may influence protein stability. The negative charge in position 514 is part of an amphiphilic alpha helix and is away from the hydrophobic core of the TMS (supplementary Fig. S2f), suggesting that it may interact directly with the substrate or with other helices by creating ionic bridges.

Amino acid D537 is in the same extracellular loop as D529 (Fig. 1A) and it may also be implicated in substrate recognition. For this position, an acidic amino acid is essential for FT1 activity (Table 1). The D124 position is 100% conserved in all FBTs. Changing this universally conserved residues to neutral amino acids led to an inactive protein but making a D124E protein restored some transport activity, but of all our active mutated proteins, this is the mutated version with the most reduced capacity to transport FA (Table 1) further underscoring the importance of D124. According to the topology (Fig. 1A) position 124 would be in an intracellular loop. While the positive charge of position 133 is not essential, the positive charge of R134 is essential. Its localisation in the same intracellular loop as D124 may suggest a role in substrate translocation in the cytosol or an interaction with the membrane lipids to stabilise a structure necessary for substrate translocation.

Amino acids, D179 and E565 are essential for FT1 function since mutation for neutral or charged amino acids led to FT1 versions not transporting FA. While D179E cannot transport FA under our experimental conditions (Table 1) we could observe a reproducible partial sensitization to MTX which we could not observe with E565D. This is due to the very high concentration of MTX used for these assays. Indeed, when we increased FA concentration (from 115 nM to 1 μM) in our accumulation assay we could also see some transport in mutant D179E. Position D179 is within TMS 4 (Fig. 1) and similarly to D514 is part of an amphiphilic alpha helix (supplementary Fig. S2). Position E565 is conserved in 100% of FBTs. Its intracellular localisation between TMS 12 and 13 suggest either an important structural role or a role in the translocation of the substrate from the membrane to the cytosol.

Our data suggest a hypothetical sequence of events for FA transport. FA is likely to be first recognized by an extracellular loop in FT1. It is possible that the extracellular loop between TMS 11 and 12 is involved. Indeed, it contained two conserved amino acids D529 and D537 and mutations of these amino acids change the affinity to FA (Table 2). Transport of FA through the membrane may occur through pores created by amphiphilic alpha helices. The two amino acids D179 and D514 are both predicted to be in TMS (4 and

11); the negative charge of both positions is essential, and both aspartic acids are within amphiphilic alpha helixes that are away from the hydrophobic core of the TMS (supplementary Fig. S2). It is thus possible that these amino acids participate in FA translocation through the lipid bilayer. A number of charged conserved amino acids are within intracellular loops and the charges of D124, R134 and E565 are essential while the change in R497 is important. We hypothesize that these amino acids may be implicated in the translocation of the substrate in the cytosol. D124E was the less active of the mutant protein; E565 cannot be mutated; R134K led to a reduced affinity to FA (Tables 1 and 2); and R497 was shown to bind the substrate (Fig. 7). Further work should concentrate on topological studies and on the key residues recognizing specifically the substrate to further substantiate our proposed model.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.07.019.

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