Functional Studies of Threonine 310 Mutations in Glut1

T310I IS PATHOGENIC, CAUSING Glut1 DEFICIENCY*

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We have previously reported on a patient with the Glut1 deficiency syndrome (Online Mendelian Inheritance in Man number 606777) carrying a heterozygous T310I missense mutation in the GLUT1 gene (Klepper, J., Wang, D., Fischbarg, J., Vera, J. C., Jarjour, I. T., O'Driscoll, K. R., and De Vivo, D. C. (1999) Neurochem. Res. 24, 587–594). To investigate the molecular basis for the associated functional deficit, we constructed T310A, T310S, and T310I human GLUT1 mutants for expression in Xenopus laevis oocytes via cRNA injection. For all mutants, glucose transport was decreased, and osmotic water permeability ($P_f$) was increased. $K_m$ values for 3-O-methylglucose (3-OMG) uptake under zero-trans influx and equilibrium exchange influx conditions were, respectively, 13 ± 1 and 68 ± 5 mM for wild-type Glut1, 5 ± 1 and 25 ± 6 mM for T310A, 6 ± 3 and 30 ± 6 mM for T310I, and 5 ± 1 and 48 ± 5 mM for T310S. Compared with wild-type Glut1, we determined the following. (a) Zero-trans and equilibrium exchange influx values of 3-OMG were significantly decreased, respectively, 15 and 5% in T310A, 8 and 3% in T310I, and 40 and 34% in T310S mutants. (b) Zero-trans efflux of 3-OMG and dehydroascorbic acid uptake were significantly decreased in mutants. (c) The relative $P_f$ values for T310A, T310I, and T310S were increased 3-, 4.8-, and 3.5-fold compared with wild-type values. We found a very high negative correlation between the rate of glucose uptake and $P_f$ ($r = -0.86$), and between hydropathy and uptake ($r = -0.92$), a moderate correlation between hydropathy and $P_f$ (0.73), and a minimal correlation between uptake, $P_f$, and molecular weight. These findings are consistent with a central role for hydropathy rather than size at position 310 of this mutation.

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1 The abbreviations used are: BBB, blood-brain barrier; DHA, dehydroascorbic acid; 3-OMG, 3-O-methylglucose; $P_f$, osmotic water permeability; WT, wild-type.
Since 1991, Glut1 deficiency syndrome is due to impaired facilitated glucose transport across the BBB. Glut1 immunoreactivity in erythrocyte membranes may be normal or 50% reduced (22, 23). Fluorescence in situ hybridization and mutational analysis of the GLUT1 gene has revealed either hemizygosity or heterozygosity for missense, nonsense, insertion, deletion, and splice site mutations (24).

The desired fragment was obtained previously on a 6-year-old girl with infantile seizures and hypoglycorrhachia (cerebrospinal fluid glucose 27 mg/dl, blood glucose 83 mg/dl), and decreased erythrocyte 3-OMG uptake (46% of control) (23). The erythrocyte Glut1 immunoreactivity was normal. Direct DNA sequencing identified a missense mutation, T310I (23). Here, we report the pathogenic basis for this mutation using Xenopus laevis oocyte expression of wild-type Glut1 and its mutants T310A, T310S, and T310I. Our studies show that the size and hydrophilicity of the side chain at position 310 play an important role in Glut1 functioning. Our results reaffirm that T310I is a pathogenic mutation causing Glut1 deficiency and represent the first report of functional and kinetic studies on a disease-causing missense mutation located in the wall of the predicted glucose channel.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and cRNA Preparation from cDNA Templates—A 1913-base pair EcoRI/HindIII GLUT1 cDNA fragment derived from pcDNA3 (Invitrogen) was subcloned into a customer plasmid (pM) containing fragments of 5' and 3'-untranslated regions of Xenopus β-globin cDNA.** The desired segment was digested with EcoRI, filled in with 

**cRNAs were quantified by incorporation of a [3H]UTP tracer in the NotI site using an mMessage mMachine kit (Ambion, Austin, TX).**

**StuI and MunI sites of pM-GLUT1.** The presence of the mutations

**primers for 35 cycles with denaturation at 95°C**

**GCTGGCATACACAGG.** The three boldfaced nucleo-

**CCTGAGCCAAT**

**and T310S forward, CCTGTGTATGCC**

**ATTGGCTCAGGTATCG-**

**GGCATACACAGG; T310I reverse, GAA-

**GGCATACACAGG; T310I forward, CCTGTGTATGCC**

**CCAAT**

**GGC**

**A-T**

**GGCC and T310A reverse, GAAGGGC CGTGTTGACGATACCTGAG-**

**wild-type cRNA, or 50 nl of mutant cRNA (1 ng/nl) and kept in Barth’s solution, incubated overnight at 18°C.**

**transport rates determined in oocytes expressing mutant and wild-type Glut1.** Oocyte net transport rates were shown.

**Zero-trans influx of 3-OMG and deoxyglucose, equilibrium exchange**

**influx of 3-OMG, and zero-trans influx of DNA into Xenopus oocytes were determined as described previously (20, 27). Zero-trans influx of 3-OMG into Xenopus oocytes was performed by injecting different amounts of [14C]-labeled 3-OMG together with unlabeled 3-OMG to reach calculated concentrations of 1–50 μm in oocytes expressing wild-type and mutant T310I Glut1 (assuming a 0.5-μl water space in an average oocyte) (28). We chose the injection procedure, because the T310I mutant has a very limited glucose transport capacity. After injection, immediately before the efflux assay, oocytes were quickly washed three times in 2 ml of Barth’s solution and transferred to a scintillation vial containing 0.5 ml of Barth’s solution. After 2 min, the oocyte was transferred to another scintillation vial, and then 0.5 ml of 0.1% SDS was added to both vials and mixed by vortex. Five microliters of HionicFluor (Nuclear Data, Pleasantville, NY) were added before counting. The measured amount of glucose doubled on both sides was used to evaluate the actual intracellular glucose concentration during the efflux measurement. The efflux rate from control oocytes (oocytes injected with water instead of cRNA) was subtracted for the calculations for both the wild-type and T310I mutant-expressing oocytes.

**Confocal immunofluorescence microscopy permitted detection of Glut1 in frozen X. laevis oocyte sections as described by García et al. (29).** Goat polyclonal IgG (Glut1 C20) (Santa Cruz Biotechnology, Santa Cruz, CA) was used as primary antibody, and Alexa Fluor488-conjugated donkey anti-goat IgG (Molecular Probes, Eugene, OR) was used as the secondary antibody. Confocal microscopy was performed at the Optical Microscopy Facility at Columbia University.

**Membranes from injected oocytes (same set of oocytes used for uptake studies) were prepared by a modified method (27, 30). Briefly, 50 μl of cRNA was injected into oocytes to obtain maximum Glut1 expression and minimize any effect resulting from the variations in the amount of injected cRNA. Zero-trans influx of Glut1 in Xenopus oocytes was performed as described previously (27).**

**Western blot analysis was performed as described previously (27). Briefly, 15 μl of the prepared total membrane or the purified plasma membrane samples from wild-type, mutant, and H2O-injected oocytes were subjected to 4–20% SDS-polyacrylamide gradient gel electrophoresis.** Glut1 signals were quantified digitally using a densitometer equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The densities of wild-type Glut1 signals in purified oocyte membranes were used to normalize mutant Glut1 densities.

**Osmotic water permeability (Pf) measurements were done as described previously (6, 20).** In brief, oocytes were placed in a glass bottom chamber at room temperature. The oocyte equatorial cross-section was viewed with a Nikon inverted microscope equipped with a 4× objective and a video camera (model NC-65, Dage-MTI, Michigan City, IN). A computer with frame grabber digitized the oocyte image and calculated the oocyte area and volume every 6 s. For an experiment, oocytes were superfused with isotonic Barth’s solution (178 mmol) for a period of 60 s and then with hypertonic solution (15 mosm, obtained by omitting NaCl) for another 100 s. Pf values were calculated from the induced change in intracellular volume, i.e., $P_f = (dV/dt)/(1/A_v(\Delta C_v))$, where $A_v$ is the area (assumed spherical) of the oocyte, $dV/dt$ is the rate of volume change, $\Delta C_v$ is the osmolarity gradient (all three at zero time), and $V_w$ is the volume of the isotonic medium (31).

**Transport kinetics were analyzed by fitting data points to the Michaelis-Menten equation utilizing Origin 228 software (OriginLab Software, Inc., Northampton, MA). Statistical analysis was done by performing t tests.**

**RESULTS**

To confirm the activity of the expressed wild-type Glut1 transporter, oocytes were injected with increasing amounts of Glut1 cRNA to determine their transport function and plasma membrane expression. A plateau is evident above 15 ng of injected cRNA. In the following kinetics experiments, 50 ng of cRNA was injected into oocytes to obtain maximum Glut1 expression and minimize any effect resulting from the variations in the amount of injected cRNA. Zero-trans influx of
3-OMG (5 mM 3-OMG in the medium) into oocytes was also determined as a function of the incubation time and showed that influx was relatively linear for about 5 min. We used 2 min as the standard incubation time for all experiments.

The studies of Hebert and Carruthers (32) with purified erythrocyte glucose transporters (Glut1) suggest that this protein may exist as a dimer as well as a tetramer. To address this question, we co-injected T310I mutant and WT Glut1 cRNA (T) or with a mix of wild-type and T310I cRNA at ratios of 1:1, 1:2, and 1:4. Three days later, the zero-trans influx of 3-OMG (5 mM) into oocytes was determined. Values represent the mean ± S.E. In another group, oocytes were injected with 1.5 and 3 ng of wild-type, 1.5 and 3 ng of T310I cRNA, or a mix of wild-type and T310I cRNA at a ratio of 1:1 (1.5/1.5 ng). Three days later, the zero-trans influx of 3H-labeled 2-deoxyglucose (DOG) (2 mM) into oocytes was determined. Symbols represent the mean ± S.E. This figure represents one of the three independent experiments.

Significantly higher than those for the mutants (all p values < 0.0001) (Table I).

Importantly, similar to what we found for glucose transport by Thr-310 mutants, the zero-trans influx of DHA was slightly facilitated compared with water-injected controls, but was significantly decreased compared with wild-type Glut1 (Fig. 4). The relative values of DHA uptake by the oocytes expressing wild-type Glut1 and mutants are Thr > Ser > Ala > Ile, similar to the order seen with glucose transport (Fig. 4) (DHA concentration ranges from 50 to 800 μM).

To show that mutant Glut1 transporters are targeted to the plasma membrane, confocal microscopy of Xenopus oocytes expressing wild-type, T310A, T310I, and T310S mutant Glut1 was performed (Fig. 5A). Western blot analysis of the total membrane and purified plasma membrane is shown in Fig. 5B. Also, the relative amounts of T310A, T310I, and T310S mutant Glut1 compared with the wild-type Glut1 immunoreactivity in plasma membrane and total membrane and the ratio of Glut1 in purified plasma membrane compared with total membrane are shown in Table II. The V_max values of mutants (Table I) normalized by the relative amount of Glut1 in purified plasma membrane were then compared with that of wild-type Glut1 to estimate the relative transport activity (Table II).

The decrease of transport activity in oocyte-expressing mutant Glut1 (Fig. 2) could be due to a defect of the transporter itself or the decreased incorporation of Glut1 into the plasma membrane. Western blot and confocal microscopy show (Fig. 5) that the Thr-310 mutant Glut1s can be expressed by the cell and translocated to the plasma membrane, which is equivalent to the results with wild-type Glut1 (Fig. 5, and Table II); the ratio of transporter in purified plasma membrane to total oocyte membrane for mutant Glut1s (T310A, 84%; T310I, 59%; and T310S, 79%) is very similar to that of oocytes expressing wild-type Glut1 (60%). This argues against the trapping of Glut1 in the Golgi body or degradation of the injected cRNA.
It has been shown that Glut1 has a finite permeability to water (6). We also have found that the $P_f$ of T310I Glut1 mutant is paradoxically increased (20). In these studies we used water permeability as a tool to investigate the effects of mutation on Glut1.

$P_f$ values were determined for oocytes expressing either wild-type Glut1 or its T310A, T310I, and T310S mutants. The background $P_f$ of water-injected oocytes was subtracted from all groups. These values were then normalized using the respective expression levels (Table II) of the WT and mutants in plasma membrane. The normalized $P_f$ values are shown in Fig. 6. The values for 3-OMG uptake at 5 mM under zero-trans influx condition (Fig. 2A) are also shown in Fig. 6 to emphasize the fact that all mutations result in a decrease of glucose transport and an increase in water permeability. $P_f$ values were (in $\mu$m/s) as follows: wild-type, 10.2 ± 0.9; T310S, 36.0 ± 1.2; T310A, 30.9 ± 0.9; and T310I, 48.7 ± 1.5. As can be seen, the relative $P_f$ values for T310A T310I, and T310S were 3, 4.8, and 3.5 times greater than that of the wild-type. Despite the different properties of the side chains, all three mutants decrease the glucose transport and increase the $P_f$ compared with the wild-type Glut1. We found a very high negative correlation between the rate of glucose uptake and $P_f$ ($r = -0.93$) and between hydropathy and uptake ($r = -0.92$), a moderate correlation between hydropathy and $P_f$ ($r = 0.73$), and a minimal correlation between uptake, $P_f$ and molecular weight (Table III). $P_f$ values were all significantly higher for each one of the mutants than for the wild-type (Table III).

**DISCUSSION**

*X. laevis* oocytes have been used widely to study structure-function relationships of the glucose transporter family. To study the pathogenesis of the human T310I missense mutation, we constructed an oocyte-expressing vector containing the wild-type GLUT1 cDNA with partial 5'- and 3'-untranslated regions. Oocytes injected with the synthesized cDNA from pM-
Table I

|                | Zero-trans influx of 3-OMG | Equilibrium exchange influx |
|----------------|----------------------------|----------------------------|
|                | $K_m$ (pmol/mM/oocyte) | $V_{max}$ (nM pmol/oocyte) | $K_m$ (pmol/mM/oocyte) | $V_{max}$ (nM pmol/oocyte) |
| WT             | 13 ± 1                    | 743 ± 52                   | 68 ± 5                    | 4406 ± 226                |
| T310A          | 5 ± 1                     | 157 ± 25                   | 25 ± 6                    | 318 ± 35                  |
| T310I          | 8 ± 3                     | 39 ± 3                     | 30 ± 6                    | 76 ± 8                    |
| T310S          | 5 ± 1                     | 234 ± 27                   | 48 ± 5                    | 1159 ± 71                 |

Table II

|                | Relative Glut1 in PM$^a$ | Relative Glut1 in TM$^b$ | PM/TM ratio$^c$ | Relative activity in zero-trans influx$^d$ | Relative activity in equilibrium exchange influx$^e$ |
|----------------|-------------------------|--------------------------|----------------|--------------------------------------------|--------------------------------------------------|
| WT             | 100                     | 100                      | 60             | 100                                        | 100                                              |
| T310A          | 147                     | 104                      | 84             | 15                                         | 5                                                |
| T310I          | 66                      | 65                       | 59             | 8                                          | 3                                                |
| T310S          | 78                      | 79                       | 79             | 40                                         | 34                                               |

$^a$ The relative Glut1 in the plasma membrane (PM), the relative Glut1 in the total membrane (TM), and the PM/TM ratio are calculated using the averaged digitized Glut1 densities from three Western blot tests (Fig. 5B).

$^b$ For the relative activity in zero-trans influx, the $V_{max}$ value of zero-trans influx of 3-OMG was normalized to the relative amount of plasma membrane Glut1 and then compared to the $V_{max}$ of wild-type Glut1 expressed in Xenopus oocytes.

$^c$ For the relative activity in equilibrium exchange influx, the $V_{max}$ value of the equilibrium exchange influx of 3-OMG was normalized using the relative amount of plasma membrane Glut1 and then compared to the $V_{max}$ of wild-type Glut1 expressed in Xenopus oocytes.

Fig. 5. Glut1 expression in Xenopus oocytes. A, localization of mutant Glut1 in oocytes by confocal immunofluorescence microscopy. Oocytes were injected with wild-type or mutant GLUT1 cRNAs or 50 nl of water. Three days later, the oocytes were frozen, sectioned, stained, and subjected to confocal immunofluorescence microscopy using primary antibody goat polyclonal IgG (Glut1 C20) (Santa Cruz Biotechnology) and secondary antibody (Alexa Fluor 568-conjugated donkey anti-goat IgG). B, Western blot analysis of total membrane (TM) and purified plasma membrane (PM) from Xenopus oocytes. Oocytes were injected with wild-type or mutant GLUT1 cRNAs or 50 nl of water. Three days later, total membranes and purified plasma membranes were prepared and then subjected to Western blot analysis as described under “Experimental Procedures.” Fifteen microliters of prepared membrane protein from three oocytes were loaded per lane. This figure represents one of the three independent Western blot analyses. Glut1 signals were quantified digitally using a densitometer equipped with ImageQuant software (Amersham Biosciences). The averaged densities of wild-type Glut1 signals in purified oocyte membranes were used to normalize mutant Glut1 averaged densities (Table II).

GLUT1 express fully functional Glut1 (data not shown). Oocytes also express Glut1 mutants, including the T310I mutant, which transports glucose very poorly (Table II and Fig. 5). These results allowed us to co-express wild-type and T310I mutant Glut1s to explore a possible role of oligomerization in function. The results allow us to conclude that, in Xenopus oocytes, the Glut1 monomer is the functional unit. These conclusions also confirm an earlier study of monomer functioning by Burant and Bell (33), who used the coexpression of Glut2 and Glut3 or Glut3 and incompetent Glut3-Leu-410 in Xenopus oocytes.

Thrreonine 310 is conserved throughout Glut proteins (Glut1–5) and across species (34). This hydrophilic amino acid is predicted to be near (5) or at (18) the exofacial end of helix 8, which, in turn, is predicted to be involved in the formation of the water-accessible glucose channel (17, 18, 35). The threonine hydrophilic side chain with its hydroxyl group may play an important role in forming hydrogen bonds with glucose (36).

The T310I mutation substitutes the hydrophobic amino acid isoleucine. This mutation causes a decrease (46% of control) in human erythrocyte 3-OMG transport. The erythrocyte expresses both the wild-type and the mutant Glut1s, because the human condition is transmitted as an autosomal dominant trait (23). It is not clear whether the functional differences between the Thr-310 and Ile-310 structures is due to the hydroxyl group or the size of the side chain. To address these issues, we examined the effects of two other amino acid substitutions (Ala and Ser) at position 310. All three mutations affected 3-OMG uptake (Table I), causing similar decreases in $K_m$. Serine, which has a small hydrophilic side chain, produced the least reduction in $V_{max}$ (Table II). Isoleucine, with a large hydrophobic side chain, resulted in the greatest decrease in $V_{max}$. Alanine, which has a small hydrophobic side chain, caused an intermediate decrease of $V_{max}$. In the aggregate, these results indicate a well defined optimum for both size and hydrophilicity for efficient glucose transport.

Glut1 is asymmetrically distributed on the luminal and abluminal membranes of endothelial cells in the BBB. Both influx and efflux of Glut1 are required for the transport of blood glucose across the BBB into brain cells (2, 3, 37, 38). We also observed a significant decrease of the zero-trans efflux in the T310I mutant Glut1 to ~3% of the wild-type Glut1 activity (Fig. 3). However, we are not able to determine whether the influx and efflux of the T310I mutant Glut1 are equally affected in vivo.

Vitamin C, as a cofactor for dopamine β-hydroxylase, is involved in the biosynthesis of catecholamines. Vitamin C also can inhibit the peroxidation of membrane phospholipids and act as a free radical scavenger in the brain (39, 40). Vitamin C can enter the brain as DHA facilitated by Glut1. DHA then is rapidly reduced and irreversibly trapped as ascorbic acid (10). This linked kinetic sequence establishes an increasing gradient for vitamin C between blood and brain. The vitamin C concentration in human brain is 10 times higher than the serum concentration (41). The zero-trans influx studies with DHA showed a significant decrease in the transport activity by the mutant Glut1s expressed in X. laevis oocytes (Fig. 4). This finding is consistent with the findings of reduced DHA uptake into erythrocytes isolated from Glut1DS patients (42). All the
mutations affected DHA and glucose transport similarly (see "Results" and Fig. 4), strongly supporting the idea that both substrates are transported by the same mechanism (9).

We had speculated previously that impaired DHA transport into brain may contribute to the pathophysiology of this clinical syndrome (42). Recently, a Na+-dependent l-ascorbic acid transporter (SVCT2) has been identified in human brain (43). It is not clear whether SVCT2 is expressed in the BBB. If so, the importance of DHA transport into brain by Glut1 may be less in maintaining the high vitamin C concentration in the brain (41).

We described previously that glucose competes for water passage in the wild-type Glut1 but not in its T310I mutant (20). The three-dimensional model for Glut1, derived from these experiments, had two channels in it (18), suggesting a relation between glucose and water transport based on a model that had two channels per Glut1 monomer. In the present study, the size and hydrophilicity of alanine and serine differ from isoleucine, yet all these mutants decrease glucose transport and increase water transport. These findings suggest a negative correlation that is apparent in Fig. 6. Similarly, Table III shows the high negative correlation between hydropathy and uptake (−0.92) and a moderate correlation between hydropathy and \( P_f \) (0.73). On the other hand, there is only a minimal correlation between uptake or \( P_f \) and molecular weight. How can the interactions between Glut1, glucose, and water be explained?

Widdas and Baker, arguing from experimental results and thermodynamic arguments (44, 45) have emphasized the permissive role of water for Glut1 to function. Moreover, analyzing the effects of high pressure on glucose transport, Naftalin et al. (46) have modeled glucose-water interactions with Glut1 as a thermodynamic cycle. They propose that several successive steps are involved in glucose transport; in one of them, Glut1 would change its structure and increase both its flexibility and its hydration, which, in turn, would decrease the affinity of the binding site(s) for glucose and increase glucose mobility inside the protein.

The results we are describing here are consistent with the proposals by Widdas et al. (44–46). The \( K_m \) values for the mutants are significantly lower than those for the wild-type Glut1 under zero-trans influx and equilibrium exchange influx conditions (Table I). Hence, one presumes that these mutations do not disturb a main Glut1 glucose-binding site (perhaps in the QLS motif region) (18, 47) but increase the affinity of glucose binding. On the other hand, the mutations significantly decrease \( V_{\text{max}} \) (Table I), perhaps by decreasing the mobility of glucose along its channel. In other words, the mutations would drive Glut1 into a predominant state of high glucose affinity, low glucose mobility. In the terms of Naftalin et al. (46), this state (step 1) would lead to a smaller amount of bound water so that water would be more mobile inside Glut1. This is consistent with the increased \( P_f \) we find for all mutants. In this model, conversely, an increase in bound water during the active or translocation phase of the Glut1 cycle (step 2 in Naftalin et al.; Ref. 46) is accompanied by the low water permeability observed across Glut1 (6, 31, 48). In the mutants, increased water permeability would correspond to a relative decrease in water binding ability, leading to reduced Glut1 flexibility and reduced glucose transport.

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