Identification of essential amino acids for glucose transporter 5 (GLUT5)-mediated fructose transport

Karolin Ebert, Maren Ewers, Ina Bisha, Simone Sander, Tanja Raspustniac, Hannelore Daniel, Iris Antes*, and Heiko Witt*

1Pediatric Nutritional Medicine, Klinikum rechts der Isar (MRI), Else Kröner-Fresenius-Zentrum für Ernährungsmedizin (EKFZ), Technische Universität München, 85354 Freising, Germany
2Department of Biosciences and Center for Integrated Protein Science Munich, Technische Universität München, 85354 Freising, Germany.
3Nutritional Physiology, Technische Universität München, 85354 Freising, Germany

*Corresponding authors
# Authors contributed equally

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To whom correspondence should be addressed:
Prof. Dr. Iris Antes, Technische Universität München, Department of Biosciences and Center for Integrated Protein Science Munich, Emil-Erlenmeyer-Forum 8 (Geb. 4264), 85354 Freising, Phone: 0049 (0)8161-712242, E-mail: antes@tum.de
Prof. Dr. Heiko Witt, Technische Universität München, Pädiatrische Ernährungsmedizin, Gregor-Mendel-Str. 2, 85354 Freising, Phone: 0049 (0)8161-712466, E-mail: heiko.witt@tum.de

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ABSTRACT

Intestinal fructose uptake is mainly mediated by glucose transporter 5 (GLUT5/SLC2A5). Its closest relative, GLUT7, is also expressed in the intestine, but does not transport fructose. For rat Glut5, a change of glutamine to glutamic acid at codon 166 (p.Q166E) has been reported to alter substrate-binding specificity by shifting Glut5-mediated transport from fructose to glucose. Using chimeric proteins of GLUT5 and GLUT7, we here identified amino acid residues of GLUT5 that define its substrate specificity. The proteins were expressed in NIH-3T3 fibroblasts, and their activities were determined by fructose radiotracer flux. We divided the human GLUT5 sequence into 26 fragments and then replaced each fragment with the corresponding region in GLUT7. All fragments that yielded reduced fructose uptake were analyzed further by assessing the role of individual amino acid residues. Various positions in the first extracellular loop, the 5th, 7th, 8th, 9th, and 10th transmembrane domains (TMDs); and the regions between the 9th and 10th TMDs and 10th and 11th TMDs were identified as being important for proper fructose uptake.

Although the p.Q167E change did not render the human protein into a glucose transporter, MD simulations revealed a drastic change in the dynamics and a movement of the intracellular loop connecting the 6th and 7th TMDs, which covers the exit of the ligand. Finally, we generated GLUT7-GLUT5 chimera consisting of the N-terminal part of GLUT7 and the C-terminal part of GLUT5. Whereas this chimera was inactive, we demonstrate fructose transport after introduction of four amino acids derived from GLUT5.

Members of the Major Facilitator Superfamily (MFS) enable sugar transport across membranes. Amongst others, the GLUT (SLC2) family of facilitative sugar transporters provides the different monosaccharides to cells as uniporters. All 14 members share common features such as 12 putative membrane spanning helices and intracellular C- and N-termini, but differ in transport characteristics and tissue expression [1]. Fructose transport across the apical membrane of enterocytes is mediated by GLUT5, whereas GLUT2 facilitates transport across the basolateral membrane. Although other members of the GLUT
family (e.g. GLUT7, GLUT8 and GLUT12) are present in the small intestine, their expression level and contribution to overall fructose transport seems to be of minor importance [2-4]. The involvement of GLUT5 in the pathophysiology of fructose malabsorption is a matter of debate. In this condition, intestinal fructose absorption is limited leading to osmotic diarrhea combined with abdominal pain and flatulence following fermentation of non-absorbed fructose by colonic bacteria. Although GLUT5 is the only fructose transporter in the apical membrane, GLUT5 expression is not lower in affected subjects [5].

Recently, the crystal structure of rat and bovine Glut5 has been described. The authors provide evidence that a gated-pore mechanism with involvement of the 7th and 10th transmembrane domain (TMD), in addition to the previously described rocker-switch-type movement, controls transport. On basis of this crystal structure, several amino acids in the central cavity that could be involved in substrate binding were identified. However, direct fructose transport measurements were not performed [6]. Studies with GLUT2-GLUT3 chimeras indicate that the sequence between the 9th and 12th TMD determines the glucose affinity of GLUT2, whereas the region from the 7th to 8th TMD is involved in fructose recognition [7]. Analysis of GLUT5-GLUT3 chimeras revealed that the region between the N-terminus and the first intracellular loop and the sequence including the 3rd extracellular loop until the 11th TMD are important for fructose transport [8].

So far, only large regions and not individual amino acid residues have been studied as of importance in fructose transport upon functional analysis. Regarding GLUT5, only chimeras with GLUT family members of different classes were analyzed. GLUT5 and GLUT7 both belong to the class II. Although their protein sequence is approximately 60 % identical, the transport characteristics are different. GLUT5 represents a high capacity fructose transporter [2], whereas GLUT7 does not transport fructose and its physiological substrate remains unknown [9]. We thus considered that the generation of chimeras of these two transporters might be a suitable model to identify amino acids and protein domains involved in fructose transport.

RESULTS

We constructed GLUT5-GLUT7 chimeras by dividing the complete protein sequence of GLUT5 into 26 fragments which we consecutively replaced by homologous domains of GLUT7. To be able to assess whether the proteins were delivered to the cell membrane, GFP was fused onto the carboxy terminus. Protein abundance and membrane localization were tested for all chimeras by means of Western blot analysis and fluorescence microscopy. Except chimera F9, all chimeric proteins were produced properly and were detected in the plasma membrane of NIH-3T3 cells (supplemental Fig. 1 and 2).

Analysis of fructose uptake activity of the GLUT5-GLUT7 chimeras revealed several important regions that mediate fructose transport (Fig. 1). The amino acid numbers of the exchanged fragments are given in brackets. Chimeras F1 (1-18), F3 (43-48), F4 (55-73), F5 (80-96), F6 (101-111), F7 (118-141), F8 (143-162), F10 (183-197), F11 (204-225), F12 (229-241), F14 (255-271), F16 (310-321), F22 (429-447), F23 (453-473), F24 (476-487) and F26 (+5 AA) showed normal or even slightly elevated fructose uptake compared to the G5GFp wild-type protein and were not further studied. A decreased uptake ranging between 30 % and 80 % of that obtained with the wild-type proteins was observed for chimeras F2 (23-41), F13 (242-254), F17 (323-338), F18 (343-357), F19 (361-381) and F25 (488-501). Fragment 25 was finally also excluded after recording normal fructose uptake by chimera G5-429-G7 (GLUT7 sequence from amino acid 429 [corresponding to F22-F26]). A very strong reduction in fructose uptake (<30 % of wild-type GLUT5-GFP) was observed for chimeras F9 (164-181), F15 (286-305), F20 (382-399) and F21 (409-428).

The chimeras that showed intermediately reduced and drastically reduced fructose transport were further investigated. Fragments that contained many amino acid changes were divided into smaller fragments [9a (164-165), 9b (166-168), 9c (170-174), 9d (175-181), 13a (242-245) 13b (247-249) 13c (250-254), 17a (323-330), 17b (331-333), 17c (334-338), 18a (343-349), 18b (350-357), 19a (361-364), 19b (365-368), 19c (370-373), 19d (375-381), 20a (382-384), 20b (385-389), 20c (394-397), 20d (398-399), 21a (409-410), 21b (415-417), 21c (422-425) and 21d (426-428)] and after subsequent analysis further split to come to the level of individual amino acids [9a (p.P166T), 9b (p.Q167E), 9bc (p.L168V), 9ca (p.I170V), 9cb
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(p.T171I), 9cc (p.I174V), 17aa (p.A323V), 17ab (p.V326I), 17ac (p.F330I), 17ba (p.C331T), 17bb (p.A332S), 17bc (p.V333A), 19aa (p.A361V), 19ab (p.A362V), 19ac (p.A364L), 19ba (p.L365F), 19bb (p.D367N), 19bc (p.T369R), 20aa (p.S382A), 20ab (p.V384I), 20ba (p.I385A), 20bb (p.A388S), 20bc (p.L389I), 20da (p.L398V), 20db (p.I399R), 21aa (p.P409R), 21ab (p.S410A), 21ba (p.G415D), 21bb (p.S417A), 21da (p.V426I), 21db (p.L428F]). The other fragments were broken down to the amino acid level directly [2a (p.I23S), 2b (p.S29A), 2c (p.V36L), 2d (p.A37S), 2e (p.A38V), 2f (p.S41T)], 15a (p.G286A), 15b (p.V293I), 15c (p.Y297N), 15d (p.Q302T), 15e (p.L305T). Chimera s F9b, F9c, F17b, F20d, F21a, F21b, and F21d exhibited a drastically reduced fructose uptake whereas chimeras F17a, F19a, F19b, F20a and F20b demonstrated an intermediately decreased uptake of fructose. Because normal fructose uptake was shown by chimeras F13a, F13b, F13c, F18a and F18b, these fragments were not further divided (Fig. 2).

A variety of single amino acid mutants in the GLUT5 backbone as p.S41T (F2f), p.L168V (F9bc), p.I170V, p.T171I, and p.I174V). Although all mutants showed similar protein levels in the membrane, we could not detect any glucose transport activity (supplemental Fig. 3).

To test whether if the exchanges found in GLUT5-GLUT7 chimeras can affect the transport properties of GLUT7 we also constructed a series of GLUT7-GLUT5 chimeras. Here, the numbering refers to the GLUT7 sequence (+6 amino acids). Four different constructs with the N-terminal part of GLUT7 and the C-terminal part of GLUT5 (from amino acid 220 GLUT5) were analyzed. Chimera G7-220-G5 served as control and did not contain any amino acid change in the N-terminal part. G7-220-G5-A contained all exchanges found to be important for fructose transport in the N-terminal part of GLUT5 (p.L42V, p.T47S, p.E173Q, p.V174L, p.V176I and p.V180I). Only those amino acid changes that showed drastically reduced fructose uptake in GLUT5-GLUT7 chimeras were exchanged in chimera G7-220-G5-B (p.L42V, p.E173Q and p.I177T). In chimera G7-220-G5-C, the amino acids which resulted in intermediately reduced fructose uptake in GLUT5-GLUT7 chimeras were exchanged in chimera G7-220-G5-C, the amino acids which resulted in drastically reduced fructose uptake in GLUT5-GLUT7 chimeras were exchanged in chimera G7-220-G5-D (Fig. 4). We also generated 14 other GLUT7 chimeras containing variants found in the C-terminal part of GLUT5. Unfortunately, all these chimeras did not appropriately translocate to the plasma membrane hampering further functional investigations (data not shown).

The dynamics of three systems, the wild-type (WT) and two selected variants (p.Q167E and p.I174V), were studied through 200 ns-long molecular dynamics (MD) simulations. The analysis of the root mean square deviation (RMSD) of the α-carbons of the transporter from the starting structure (supplemental Fig. 4) shows a strikingly different behavior for p.Q167E (supplemental Fig. 5, left panel): the WT and the p.I174V variant do not deviate from the starting configuration for more than 2.5-3 Å, whereas p.Q167E shows RMSD values of up to 4.5 Å. Visual inspection of the trajectories revealed that the intracellular loop between the 6th and 7th TMD is particularly flexible in the p.Q167E variant. This was verified by calculating the α-carbon RMSD of the proteins excluding this loop. In that case similar RMSD
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values can be observed for all three systems (supplemental Fig. 5, right panel).

To quantify the flexibility of each amino acid during the simulations, we calculated their root mean square fluctuation (RMSF) during the simulations (supplemental Fig. 6). We observed that the p.Q167E system (red line) is much more flexible than the WT, especially concerning the loops between 3rd and 4th TMD and 6th and 7th TMD, while the WT and the p.I174V variant show similar flexibility. As anticipated by the RMSD and RMSF calculations, in p.Q167E the intracellular (IC) loop between the TMD moves dramatically during the simulation. This movement leads to a complete change of the loop position and resembles that of a lid covering the exit of the substrate into the cytosol (Fig. 5). This loss of fructose transport into the cytosol mediated by the p.Q167E variant was also functionally demonstrated in Xenopus laevis oocytes (data not shown). Furthermore, efflux of fructose from the cytosol into the surrounding medium was absent (supplemental Fig. 7).

Finally, we investigated all 12 variants, which displayed reduced fructose uptake between 30 % and 80 % (depicted in blue in Fig. 6 and 3): These variants are located in TMD 5, 7-11, in the EC loops between TMD 1-2, between TMD 9-10, and in the IC loop between TMD 10-11 (note that p.I174V was re-simulated within this new frame). It is interesting to note that the three-dimensional distribution of these residues is asymmetric with respect to the central cavity: they are all located on the same side of the transporter (Fig. 6, panels A and B). This observation raised our curiosity about the residues which drastically reduce fructose transport. Although these amino acids are located closer to the central cavity, embracing TMD 5, 7, 8, 11 and loops between TMD 1-2 and 10-11, they are still asymmetrically distributed involving only one side of the protein (Fig. 6, panel B depicted in red). The analysis of the trajectories of the 12 “mild phenotype” variants revealed that none of the mutants exerts an equally strong effect on the IC loop of TMD 6-7 as p.Q167E. However, it is possible to detect conformational changes in several helices – e.g. shifts, bends (i.e. IC part of TMD 1 or 5, helix involved in the binding of fructose), and changes in the orientation – most probably driven by the mutated residue, which is able in this way to affect the physiological dynamics of the protein. Interestingly, even far-positioned mutations are able to affect the channel geometry in an allosteric fashion, as depicted in Fig. 6, panel C for the helix 5. A more thorough and numerical analysis was performed for the last 50 ns of all 12 simulations by calculating the RMSD of the backbone of different regions (helices, loops) of the proteins (supplemental Table 1). Compared to the WT, all variants show indeed higher RMSD values in specific regions of the protein. These regions are not necessarily in structural or sequential proximity of the mutation, indicating partially strong allosteric effects. Comprehensive, the largest movements could be observed in the TMD connecting loop regions, especially those between TMD 4-5, 6-7, and 10-11, which showed remarkably higher RMSD values for the variants than for the WT - independently of the location of the mutated residue. In addition, several variants along the different TMDs are characterized by higher RMSD values (thus, conformational changes).

Supplemental Fig. 8A shows the involved loops and in supplemental Fig. 8B the conformational changes in the three loops between TMD 4-5, 6-7, and 10-11 are illustrated by the superposed representative structures of all 12 variants.

DISCUSSION

By exchanging fragments and individual amino acid residues between GLUT5 and GLUT7, we identified several amino acid positions in the sequence that are essential for function or that contribute to fructose transport. These amino acids are found in GLUT5 in the first extracellular loop, the 5th, 7th, 8th, 9th, and 10th TMD, and the regions between 9th and 10th TMD and 10th and 11th TMD. An illustration of the topology of these amino acids is given in Fig. 3. The amino acid exchanges that reduced fructose transport below 30 % of the wild-type are labeled in red, whereas those that reduced fructose uptake from 30 % to 80 % are marked in blue. For fragments 13 and 18, shown in yellow, their influence on fructose transport is unclear: fructose uptake was reduced in both chimera, but normal in the subsequently generated subfragments. We cannot exclude that the reduced uptake of fructose is caused by unspecific inactivation of the transporter due to incorrect protein folding. To test this, we generated GLUT7-GLUT5 chimeras comprising the GLUT7 sequence at the N-terminus and the GLUT5 sequence from amino acid 220. This chimera did not transport fructose. After introduction of four amino acids derived from the GLUT5 sequence in the N-
terminal part (p.T47S, p.V174L, p.V176I and p.V180I), however, this chimera transported fructose. Unfortunately, other GLUT7 chimeras containing variants found in the C-terminal part of GLUT5 did not appropriately translocate to the plasma membrane.

Previous studies with GLUT5-GLUT3 chimeras identified two regions as important for fructose transport: the region from N-terminus to the first intracellular loop and the region from the 3rd extracellular loop until the 11th TMD [8]. Another approach using chimeras between rabbit Glut1 and rat Glut5 revealed evidence that a region from the N-terminus to the 6th TMD and the intracellular C-terminus [10] is important for transport. Our data do not match with these observations. We also found important amino acid residues between the 7th and 11th TMD while the C-terminus region appeared as not relevant for function. These contradictory findings may originate from the different sizes of exchanged fragments or the different species and thus sequences that served as backbone. However, the studies using GLUT2-GLUT3 chimeras emphasized the importance of the region between the 7th and 8th TMD for fructose transport [7] and we identified 7 amino acid residues in this region that are essential for GLUT5 fructose transport.

In 2015, the crystal structure of rat (open outward-facing) and bovine (open inward-facing, on which our models are built) Glut5 was described [6]. The amino acids p.I169 (p.I170 in human GLUT5), p.I173 (174), p.Q166 (167), p.Q287 (288), p.Q288 (289), p.N324 (325) and p.W419 (420) are located in the central cavity and are conserved between rat Glut5 and human GLUT1. The residues p.Y31 (32), p.H386 (387), p.A395 (396), p.H418 (419) and p.S391 (392) also face the central cavity but are different in GLUT1. These amino acids are located in the 1st, 5th, 8th, 10th, and 11th TMD and in the loops between the 6th and 7th and between the 10th and 11th TMD. The mutants p.Y31F/p.H386F, p.Y31F/p.H418Q, p.H418Q, p.S391A, p.H386F, p.H386A, p.Q287A, p.Q166E and p.Y31F showed markedly reduced binding of fructose (<40 % of wild-type) measured with tryptophan fluorescence quenching, whereas mutants p.Q288A, p.I173A, p.Q166A, p.I169A and p.Y31A demonstrated mild reduction of fructose binding (40-90 % of wild-type) [6].

Moreover, glucose transport activity by mutant p.Q166E was proposed since glucose binding by this mutant was enhanced [6]. The three amino acids p.I169, p.I173 and p.Q166, located in the 5th TMD were also identified to be important for fructose transport by our analysis (corresponding to p.I170, p.I174 and p.Q167 in human GLUT5). However, we demonstrate no glucose uptake by variant p.Q167E. This might be explained by the differences in the measurements: Nomura and colleagues measured only the binding of glucose to the transporter using tryptophan fluorescence quenching, whereas we measured the actual transport of glucose. We observed in our molecular dynamics simulations that the p.Q167E mutation leads to a radical change of the position of the IC loop between the 6th and 7th TMD moving the loop towards the central (median) part of the transporter, from where the ligand is most likely exiting the protein to reach the cytoplasm (Fig. 5). We hypothesize that in this case the mutation triggers the IC loop to act as a "gate", or "lid", which blocks fructose release and thereby its transport. Thus, it might be possible that the p.Q167E variant results in a better glucose binding, but absent glucose transport by precluding the exit of the ligand.

We did not analyze the other amino acid variants with reduced fructose binding reported by Nomura and co-workers [6] since GLUT5 and GLUT7 are identical at these positions. Potentially, the amino acids we identified as important for fructose transport in the 5th, 7th, 8th, 10th and 11th TMD and in the first extracellular and last intracellular loop are also involved in fructose binding since these regions are dedicated to form the pore or are in close neighborhood of it. The two amino acids p.Q167 (p.A206 in GLUT9) and p.V293 (p.L332 in GLUT9) we identified as important in our analyses were already described to line the pore in a homology-based model of human urate transporter GLUT9 [11].

To gain a detailed understanding of the effect of these mutations on the protein dynamics, we performed computational studies on several systems. For this, we built the human GLUT5 model by homology modelling using the inward-facing open conformation of the bovine crystal structure (PDB code 4YB9) as a template, and then conducted molecular dynamics simulations of the wild-type protein and of several variants. Analysis of the obtained MD trajectories revealed that all 12 mutations that resulted in a reduction of fructose transport between 30 % and 80 % (marked in blue in Fig. 6) lead to conformational changes in
different regions of the protein (especially in loops 4-5, 6-7 and 10-11) with respect to WT. We were able to observe different bends or shifts of specific loops or helices (for example TMD 5, involved in fructose binding). Remarkably, these effects were also observed in protein regions far from the mutated residue, as in the case of p.T368R mutant, demonstrating an allosteric propagation of structural changes through the protein. A deeper understanding of these effects would be beneficial in the future in order to uncover the exact mechanism underlying these mutations.

In summary, we identified residues in GLUT5 which are critical for its function as a fructose transporter and demonstrate the role of selected residues within the protein structure for the fructose transporter and demonstrate the role of selected residues within the protein structure for the dynamics via molecular simulations. Of special interest is the change in the orientation of the large intracellular loop caused by exchange of p.Q167E.

**EXPERIMENTAL PROCEDURES**

**Molecular cloning.** For all cloning procedures we used the vector pMXs with human GLUT5-GFP or GLUT7-GFP (GLUT7: NM_207420.2, GLUT5: NM_003039.2) insert as described elsewhere [9]. In the first round, we divided GLUT5 into 26 fragments covering the complete protein sequence and constructed GLUT5-GLUT7-GFP chimeras by overlap extension PCR. Each GLUT5 chimera contained one fragment with the corresponding amino acids of GLUT7 based on sequence alignment. Selected fragments, which showed altered fructose transport, were further sub-divided into smaller fragments or broken down to the amino acid level. Primer sequences are shown in supplemental Table 2. As flanking primers we used for all chimeras 5’-TTAGTTCTCGAGCTTTTGGAGTACGTCG-TCTTTAGG-3’ and 5’-AGCTAGTTAATTAAGGATCTTCCCCAGCATGCCGTGC-3’, which contain the vector sequence with cleavage sites for Xhol (F) and PacI (R). Overlap extension PCR was also applied for construction of GLUT7-GLUT5-GFP chimeras. One primer pair was not sufficient because GLUT7 had to be changed at multiple sites. Therefore, the product from one overlap extension PCR was used as new template for the next PCR. The same flanking primer as for GLUT5-GLUT7-GFP chimeras were used. Mutagenesis primers are depicted in supplemental Table 3. All primers were purchased from TIB MOLBIOL (Berlin, Germany).

After cloning, all vectors were monitored by DNA sequencing. For *Xenopus laevis* oocyte experiments, the p.Q167E variant and the G5GFP wild-type were sub-cloned in pGEM-HE vector for cRNA synthesis with the mMESSAGE mMACHINE® T7 Transcription Kit (Ambion®, Life Technologies).

**Cell culture.** NIH-3T3 mouse fibroblasts were cultured in DMEM (4.5 g/l glucose) with 10 % FBS and 1 % penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin). Additionally, 1 µg/ml puromycin and 10 µg/ml blasticidin were added for culturing of Platinum E cells.

**Retroviral transduction.** Platinum E cells were transfected with 5 µg pMXs vector with the sequence of interest. One day before transfection, Platinum E cells were split with a ratio of 1:3. Six-well plates were coated with 5 µg/cm² collagen the same day. Three to five hours after seeding of 10⁶ cells/well in medium without puromycin and blasticidin, we transfected cells using the ProFection® Mammalian Transfection System (Promega). The virus containing supernatant was collected and filtered through a 0.45 µm cellulose acetate syringe filter (Sartorius) ~16 h after transfection. Fresh medium was added to the cells. For infection of NIH-3T3 cells, we applied the filtered supernatant from day 2 after transfection. Cells were seeded into 6-well plates at a density of 60,000 cells/well and were infected with the virus containing supernatant in presence of 2 µg/ml Polybrene® the next day (20-30 % confluence). Successfully infected cells were selected with 10 µg/ml blasticidin one day after infection and cultured for further experiments.

**Radiotracer flux assay with NIH-3T3 cells.** NIH-3T3 cells were seeded out in 24-well plates and were grown to confluence. The uptake solution contained ¹⁴C-D-glucose (2 µCi/ml) or ¹⁴C-D-fructose (5 µCi/ml) (American Radiolabeled Chemicals, ARC-0122D, ARC-0116A) and the corresponding non-radiolabeled sugars (final concentration 1 mM) diluted in uptake buffer (140 mM NaCl, 20 mM HEPES, 1.7 mM KCl, 1.5 mM KH₂PO₄, 0.9 mM CaCl₂, 0.8 mM MgSO₄, pH 7.4). Cells were washed 3 times with 400 µl uptake buffer and were incubated with 200 µl uptake solution for 1 minute. After three washing steps with 400 µl ice-cold uptake buffer, cells were lysed in 200 µl 0.1 N NaOH. The cell lysate was...
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Transferred to a scintillation vial, shaked for at least 30 minutes and mixed with 3 ml Rotiszint® (Carl Roth). To normalize the influx against the protein concentration, the influx per µg protein was calculated by using one additional well per cell line for protein measurement by the Bradford assay. For this purpose, cells were washed once with 400 µl PBS and lysed completely in 200 µl 0.1 N NaOH using a sonicator.

Protein extraction from NIH-3T3 cells. Cells were seeded out in 6-well plates (3 wells per cell line) and were grown to confluence. Cells were scraped off in 1 ml ice-cold PBS after washing and were centrifuged for 2 minutes at 600 x g and 4°C. The cells were lysed in 90 µl RIPA lysis buffer (150 mM NaCl, 10 mM TRIS-HCl, 5 mM EDTA, 1 % Triton-X-100, 1 % protease inhibitor cocktail, pH 7.4) by drawing up and down with a 24 g syringe. The supernatant was collected after centrifugation for 3 minutes at 400 x g and 4°C. The pellet was extracted again using 45 µl RIPA lysis buffer. The supernatant was combined with that of the first extraction (total protein).

Chimera overexpression in Xenopus laevis oocytes. Oocytes were removed surgically from anaesthetized frogs, washed in ORII solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM HEPES, 3 mM TRIS, 6 mM pyruvate, 0.01 % gentamicin, pH 7.4) and placed in ORII-solution containing 2 mg/ml collagenase for 90 minutes. Oocytes were washed 7 times each in ORII-solution and Barth’s solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM HEPES, 3 mM TRIS, pH 7.4). Mature oocytes (stage V and VI) were stored in Barth’s solution with 6 mM pyruvate and 0.01 % gentamicin at 17°C. The following day, oocytes were injected with 12.9 ng cRNA (using an Auto-Nanoliter Injector (Nanoject II™, Drummond Scientific). Subsequent experiments were performed 4 days after injection.

Radiotracer influx assay with Xenopus leavis oocytes. To measure fructose transport into oocytes with radiotracer influx assay, we used Barth’s solution without pyruvate and gentamicin. Groups of 10 oocytes were incubated with 200 µl Barth’s solution containing 14C-D-fructose (5 µCi/ml) (American Radiolabeled Chemicals, ARC-0116A) and non-radiolabeled fructose for 10 minutes in a final fructose concentration of 1 mM. Oocytes were washed 4 times with ice-cold Barth’s solution. Single oocytes were solved in 100 µl 10 % SDS at 50°C while shaking. The solution was mixed with 3 ml Rotiszint® (Carl Roth). Radioactivity was determined with the Tri-Carb 2810 TR scintillation counter (Perkin Elmer).

Radiotracer efflux assay with Xenopus leavis oocytes. Fructose transport from the oocytes into the surrounding medium (Barth’s solution) was measured with radiotracer efflux assay. For this, oocytes 4 days after injection with cRNA were incubated with 18.4 µl fructose solution (final fructose concentration 250 mM, 25µCi/ml in Barth’s solution). Groups of 10 oocytes were incubated for 0, 15, 30, 45 or 60 minutes and were washed twice with ice-cold Barth’s solution. Single oocytes were treated as described above.

Protein extraction and paraffin embedding of Xenopus laevis oocytes. After homogenization of 30 oocytes with 200 µl lysis buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 25 mM PMSF) using a homogenizer (Polymtron® 1600 E, Kinematica), samples were centrifuged at 4 °C and 20,000 x g for one minute. The supernatant was purified in two other centrifugation steps for 2 minutes. For fluorescence pictures, 5 oocytes were fixed in 4 % PFA, dehydrated with 70 %, 80 %, 96 % and 100 % ethanol and xylol and embedded in paraffin. Paraffin blocks were cut into 6 µm slices using a HM 355S microtome (Thermo Fisher Scientific). Slices were placed on microscope slides and incubated in 100 % xylol and 100 %, 96 % and 80 % ethanol for removal of paraffin and rehydration. Afterwards, the slices were covered with Roti®-Mount FluorCare (Carl Roth).

Western blotting. Extracts (5 µg for NIH-3T3, 10 µg for oocytes) were separated on a 10 % polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in PBS with 5 % BSA and incubated with GFP (Rockland Immunochemicals; 600-401-215; 1:25,000 dilution) and actin (Santa Cruz Biotechnology; sc-1615; 1:800) specific antibodies overnight at 4 °C. Fluorescent-dye-labelled secondary antibodies (anti-rabbit IRDye® 680RD and anti-goat IRDye® 800RD, LI-COR® Biosciences, diluted 1:10,000 in PBST) were used for detection with the Odyssey® Infrared Imager (Licor).

Fluorescence microscopy. GFP fluorescence was visualized with 480 nm excitation and 505 nm emission wavelength at 22°C using the Leica Microsystems DMI 4000 B microscope.
DFC490 camera and LAS V3.8.0 software (Leica Camera AG).

**Statistical analysis.** The Software SPSS Statistics 23 (IBM) was used for statistical analysis. Only p-values <0.01 are shown.

**Molecular simulations.** The model of human GLUT5 was built with the MODELLER program [12,13] using as template the inward-facing open conformation of the bovine crystal structure (PDB code 4YB9 [6], 89.6% of sequence similarity). We performed the sequence alignment with the HH-Pred program [14] and the evaluation of the 200 models with the QMEAN server [15]. The chosen model was afterwards embedded in a pure, pre-equilibrated 1-palmitoyl-2-oleylphosphatidylcholine (POPC) lipid model membrane (kindly provided by T. A. Martinek [16]) using the gmembed tool of GROMACS [17] and the protein was oriented following the OPM database model [18]. Subsequently, the system was neutralized and solvated with TIP3P water molecules (92772 total atoms, box size of 94.1 x 92.7 x 105.5 Å³).

Simulations were carried out with the GROMACS4 [19] package using the Amber03 [20] force field for the protein and the GAFF force field [21] together with the parameters supplied by T. A. Martinek [16] for the membrane. The system was neutralized and minimized before the equilibration was performed, which consisted of three stages: (1) heating up of the system for more than 1 ns with the protein backbone completely fixed, while the side chains were left free to move; (2) 5 ns of simulation (with backbone restraints) in a NpT ensemble with a surface tension equal to 600.0 bar nm [16]; (3) 40 ns of simulation keeping the backbone restrained and the membrane area constant. Finally, three 200 ns-long molecular dynamics (MD) simulations were performed on three different systems: wild-type (WT), p.I174V (magenta residue shown in Fig. 6A) and p.Q167E (red residue shown in Fig. 6A). For the two variants, the final conformation from step (3) was mutated, minimized and equilibrated for further 20 ns of restrained MD before simulating it freely. Additionally, 100 ns of MD simulations were performed for each of the following variants: p.S41T, p.L168V, p.I170V, p.I174V, p.V293I, p.A323V, p.C331T, p.A362V, p.A364L, p.T368R, p.A388S, and p.L398V (note that p.I174V was indeed re-simulated within this new frame). All variants are based on the conformation representing the biggest cluster of the last 50 ns of the WT-MD, mutating one by one all the „blue“ residues as depicted in Fig. 6.

All the simulations were performed using periodic boundary conditions at 310 K and the Nose-Hoover thermostat [22] and Parrinello-Rahman barostat [23] with a semi-isotropic pressure coupling type and a time step of 2 fs. Position restraints of atoms were fixed with a force constant (K) equal to 1000 kJ mol⁻¹ nm⁻². This protocol has already shown to be successful before [24,25].

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**Conflict of Interest:** The authors declare no conflict of interest.

**Author contributions:** KE and ME conducted cloning and functional analysis of the chimeras, IB performed molecular dynamics (MD) simulations, SS and TR contributed to cloning and flux assays, HD provided scientific input, IA supervised MD simulations, HW supervised the study. All authors contributed to writing of the manuscript.

**REFERENCES**

1. Mueckler, M., and Thorens, B. (2013) The SLC2 (GLUT) family of membrane transporters. *Mol. Asp. Med.* **34**, 121–138
2. Burant, C. F., Takeda, J., Brot-Laroche, E., Bell, G. I., and Davidson, N. O. (1992) Fructose transporter in human spermatozoa and small intestine is GLUT5. *J. Biol Chem.* 267, 14523–14526

3. Patel, C., Douard, V., Yu, S., Gao, N., and Ferraris, R. P. (2015) Transport, metabolism, and endosomal trafficking-dependent regulation of intestinal fructose absorption. *FASEB J.* 29, 4046–4058

4. Cheeseman, C. I. (1993) GLUT2 is the transporter for fructose across the rat intestinal basolateral membrane. *Gastroenterology* 105, 1050–1056

5. Wilder-Smith, C. H., Li, X., Ho, S. S., Leong, S. M., Wong, R. K., Koay, E. S., and Ferraris, R. P. (2014) Fructose transporters GLUT5 and GLUT2 expression in adult patients with fructose intolerance. *United European Gastroenterol. J.* 2, 14–21

6. Nomura, N., Verdon, G., Kang, H. J., Shimamura, T., Nomura, Y., Sonoda, Y., Hussien, S. A., Qureshi, A. A., Coincon, M., Sato, Y., Abe, H., Nakada-Nakura, Y., Hino, T., Arakawa, T., Kusano-Arai, O., Iwanari, H., Murata, T., Kobayashi, T., Hamakubo, T., Kasahara, M., Iwata, S., and Drew, D. (2015) Structure and mechanism of the mammalian fructose transporter GLUT5. *Nature* 526, 397–401

7. Wu, L., Fritz, J. D., and Powers, A. C. (1998) Different functional domains of GLUT2 glucose transporter are required for glucose affinity and substrate specificity. *Endocrinology* 139, 4205–4212

8. Buchs, A. E., Sasson, S., Joost, H. G., and Cerasi, E. (1998) Characterization of GLUT5 domains responsible for fructose transport. *Endocrinology* 139, 827–831

9. Ebert, K., Ludwig, M., Geillinger, K. E., Schoberth, G., Essenwanger, J., Stolz, J., Daniel, H., Witt, H. (2017) Reassessment of GLUT7 and GLUT9 as putative fructose and glucose transporters. *J. Membr. Biol.* (doi: 10.1007/s00232-016-9945-7)

10. Inukai, K., Katagiri, H., Takata, K., Asano, T., Anai, M., Ishihara, H., Nakazaki, M., Kikuchi, M., Yazaki, Y., and Oka, Y. (1995) Characterization of rat GLUT5 and functional analysis of chimeric proteins of GLUT1 glucose transporter and GLUT5 fructose transporter. *Endocrinology* 136, 4850–4857

11. Clémençon, B., Lüscher, B. P., Fine, M., Baumann, M. U., Surbek, D. V., Bonny, O., and Hediger, M. A. (2014) Expression, purification, and structural insights for the human uric acid transporter, GLUT9, using the Xenopus laevis oocytes system. *PLoS ONE* 9, e108852

12. Šali, A., and Blundell, T. L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 234, 779-815

13. Martí-Renom, M. A., Stuart, A. C., Fiser, A., Sánchez, R., Melo, F., and Šali, A. (2000). Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct.* 29, 291-325

14. Söding, J., Biegert, A., Lupas, A.N. (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res.* 33 (suppl 2), W244-W248

15. Benkert, P., Künzli, M., Schwede, T. (2009). QMEAN Server for Protein Model Quality Estimation. *Nucleic Acids Res.* 37(Web Server issue), W510-4

16. Jojart, B., Martinek, T.A. (2007) Performance of the general amber force field in modeling aqueous popc membrane bilayers. *J. Comput. Chem.* 28, 2051–2058

17. Wolf, M.G., Hocflying, M., Aponte-Santamarıa, C., Grubmueller, H., Groenhof, G. (2010) gmemb: Efficient insertion of a membrane protein into an equilibrated lipid bilayer with minimal perturbation. *J. Comput. Chem.* 31, 2169–2174

18. Lomize, M.A., Lomize, A.L., Pogozheva, I.D., Mosberg, H.I. (2006) Opm: orientations of proteins in membranes database. *Bioinformatics* 22, 623–625

19. Hess, B., Kutzner, C., van der Spoel D., Lindahl, E. (2008) Gromacs 4, Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theory Comput.* 4, 435–447

20. Duan, Y., Wu, C., Chowdhury, S., Lee, M. C., Xiong, G., Zhang, W., Yang, R., Cieplak, P., Luo, R., Lee, T., Caldwell, J., Wang, J., and Kollman, P. (2003) A point-charge force field for molecular mechanics simulations of proteins based on condensed- phase quantum mechanical calculations. *J. Comput. Chem.* 24, 1999–2012

21. Wang, J., Wolf, R., Caldwell, J., Kollman, P., Case, D. (2004) Development and testing of a general amber force field. *J. Comput. Chem.* 25, 1157–1174

22. Evans, D. J., and Holian, B. L. (1985) The nose–hoover thermostat. *J. Chem. Phys.* 83, 4069-4074
23. Nosé, S., and Klein, M. L. (1983) Constant pressure molecular dynamics for molecular systems. *Molecular Physics* **50**, 1055-1076

24. Bisha, I., Rodriguez, A., Laio, A., and Magistrato, A. (2014) Metadynamics simulations reveal a Na+ independent exiting path of galactose for the inward-facing conformation of vSGLT. *PLOS Comput. Biol.* **10**, e1004017

25. Napolitano, L. M. R., Bisha, I., De March, M., Marchesi, A., Arcangeletti, M., Demitri, N., and Laio, A. (2015) A structural, functional, and computational analysis suggests pore flexibility as the base for the poor selectivity of CNG channels. *Proc. Natl. Acad. Sci. U.S.A.* **112**, E3619-E3628
FIGURE LEGENDS

Figure 1. Fructose uptake into NIH-3T3 cells by GLUT5-GLUT7-GFP chimeras (large fragments). GFP control, GLUT5-GFP and GLUT5-GLUT7-GFP chimera cells were incubated with 1 mM fructose for 1 minute. Bars represent mean values of 6 wells as percentage of GLUT5-GFP after subtraction of GFP control values. Error bars indicate the standard deviation. Mann-Whitney-U-test was used to test for statistical significance compared to GLUT5-GFP (**p<0.01).

Figure 2. Fructose uptake into NIH-3T3 cells by GLUT5-GLUT7-GFP chimeras (smaller fragments and single amino acid changes). GFP control, GLUT5-GFP and GLUT5-GLUT7-GFP chimera cells were incubated with 1 mM fructose for 1 minute. Bars represent mean values of 6 wells as percentage of GLUT5-GFP after subtraction of GFP control values. Error bars indicate the standard deviation. Mann-Whitney-U-test was used to test for statistical significance compared to GLUT5-GFP (**p<0.01).

Figure 3. Illustration of exchanged fragments and amino acids in GLUT5-GLUT7 chimeras. Amino acid changes that resulted in decreased fructose transport are shown in blue (30-80 %) or red (<30 %) whereas green dots represent amino acid exchanges that did not influence fructose transport. White circles indicate amino acids which are identical in GLUT5 and GLUT7. Fragment 13 and 18 are illustrated in yellow since exchange of the whole fragment reduced fructose transport but exchange of smaller fragments did not. Squares indicate additional amino acids of GLUT7, since GLUT7 compromises of 512 amino acids compared to 501 amino acids of GLUT5. In total, GLUT7 has 6 additional amino acids at the N- and 5 additional amino acids at the C-terminus. All domains besides the 1st and 7th intracellular domain are assumed to be similar in length.

Figure 4. Fructose uptake into NIH-3T3 cells expressing GLUT7-GLUT5-GFP chimeras. Stable cell lines were created using retroviral transduction. (A) GFP-control, GLUT5-GFP, G7-220-G5, G7-220-G5-A, G7-220-G5-B and G7-220-G5-C fibroblasts were incubated for 1 minute with 1 mM fructose. Values of GFP-control cells were subtracted and % of wild-type GLUT5-GFP was calculated. Bars represent mean values of 11-12 wells from two independent experiments. Error bars indicate the standard deviation. Mann-Whitney-U-test was used to test for significant differences compared to GLUT5-GFP (**p<0.001). (B) Western blot of NIH-3T3 protein extracts (5 µg per lane) stained with GFP (red) and actin (green) antibodies. (C) GFP fluorescence was visualized using a Leica microscope (10x magnification).

Figure 5. Movement of IC loop of TMD 6-7 in the WT (cyan) and p.Q167E (red) systems. (A) Side view with the protein in surface representation (IC loops of two systems in opaque colors, the rest of the protein in transparent cyan) and the membrane in surface and stick representation (gray); (B) bottom view with the protein in surface representation and the IC loop in cartoon. Conformations represent the most populated cluster of the last 150 ns of the two molecular dynamics simulations.

Figure 6. Localization of the analyzed variants in the GLUT5 protein. (A) Side view of the protein (cyan ribbons) and the 12 mutated amino acids, which intermediated reduced fructose uptake, in blue spheres; p.Q167 in red spheres and p.I174 in magenta spheres; the membrane is shown in gray spheres and sticks. (B) Top view showing all identified residues: residues intermediated reducing fructose uptake between 30-80 % are shown in blue and residues with an uptake reduction below <30 % in red, respectively. The blue and red dashed lines highlight their asymmetric distribution around the pore. (C) Side view of the WT-transporter (transparent gray and cyan ribbons) and TMD 5 (red) and part of TMD 4 (orange) of the p.T368R variant (after superposing of both proteins). The mutated residue is shown with yellow spheres.
Figure 3

Fructose transport of GLUT5-GLUT7 chimeras

[Diagram showing the transport of fructose through GLUT5-GLUT7 chimeras with various amino acid changes marked on the extracellular and intracellular domains.]
Figure 4

A) 

Fructose uptake (% of wild-type GLUT5-GFP)

B) 

C) 

GFP-control  GLUT5-GFP  G7-220-G5  G7-220-G5-A  G7-220-G5-B  G7-220-G5-C

200 μm
Figure 5

A) 

B)
Fructose transport of GLUT5-GLUT7 chimeras
Identification of essential amino acids for glucose transporter 5 (GLUT5)-mediated fructose transport

Karolin Ebert, Maren Ewers, Ina Bisha, Simone Sander, Tanja Rasputniac, Hannelore Daniel, Iris Antes and Heiko Witt

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