Leukocyte adhesion deficiency II patients with a dual defect of the GDP-fucose transporter

Yvonne Helmus, Jonas Denecke, Sviatlana Yakubenia, Peter Robinson, Kerstin Lühn, Diana L. Watson, Paraic J. McGrogan, Dietmar Vestweber, Thorsten Marquardt and Martin K. Wild

1Max Planck Institute of Molecular Biomedicine and Institute of Cell Biology, ZMBE, University of Münster, Münster, Germany, 2Department of Pediatrics, University Hospital of Münster, Münster, Germany, 3Yorkhill NHS Trust, Royal Hospital for Sick Children, Glasgow, UK. Kerstin Lühn’s present address is: Weatherall Institute of Molecular Medicine, MRC Human Immunology Unit, University of Oxford, Oxford, UK

* Yvonne Helmus and Jonas Denecke contributed equally to this work.

Supported by the Max-Planck Society and by the SFB 293 of the Deutsche Forschungsgemeinschaft (Y.H., K.L., D.V., M.K.W.) and by a fellowship of the International Graduate Research School “Molecular Basis of Dynamic Cell Processes”, GRK 1050 (S.Y.)

Correspondence: Martin K. Wild, Max Planck Institute of Molecular Biomedicine and Institute of Cell Biology, ZMBE, University of Münster, Von-Esmarch-Strasse 56, 48149 Münster, Germany. Phone: +49-251-83 52180; Fax: +49-251-8352236; email: wildm@uni-muenster.de or Thorsten Marquardt, Universitätsklinikum Münster, Klinik für Kinder- und Jugendmedizin, Albert-Schweitzer-Str. 33, 48149 Münster, Germany, Phone: +49-251-8358519; email: marquat@uni-muenster.de

Word counts: text, 4972; abstract, 197.

Scientific heading: Immunobiology

Short title: Dual defect in Leukocyte adhesion deficiency II
**Editorial note:** Y. Helmus, J. Denecke, and S. Yakubenia performed the research. P. Robinson, D.L. Watson, P.J. McGrogan described and attended the patient. K. Lühn contributed vital new reagents. D. Vestweber designed the research. T. Marquardt designed the research and analyzed data. M.K. Wild designed the research, analyzed data, and wrote the paper.
Abstract

Leukocyte adhesion deficiency II (LAD II) is a rare congenital disease which is caused by defective fucosylation leading to immunodeficiency and psychomotor retardation. We have previously identified the genetic defect of LAD II in a patient whose Golgi GDP-fucose transporter (GFTP) bears a single amino acid exchange that renders this protein non-functional but correctly localized to the Golgi. We now report a novel dual defect by which a truncated GFTP causes the disease in a new LAD II patient. We show that the truncation renders this GFTP unable to localize to the Golgi, the compartment where it is required. Furthermore, the missing part of the GFTP can be dissected into two regions, one that is needed for Golgi localisation and one that is additionally required for the function of the GFTP. We investigated the subcellular localisation of all known defective GFTPs allowing us to divide all genetically analysed LAD II patients into two groups, one in which single amino acid exchanges in the GFTP impair its function but not its subcellular localisation, and another group with a dual defect in function and Golgi expression of the GFTP due to the absence of two important molecular regions.
Introduction

Leukocyte adhesion deficiency II (LAD II) is a congenital disorder of glycosylation (CDG-IIc) which affects leukocyte interactions with blood vessel endothelium. These interactions are required for the extravasation of leukocytes to secondary lymphoid organs and sites of infection and involve a cascade of binding events that are initiated by selectin-dependent leukocyte tethering and rolling \(^1,2\). Selectins are a group of C-type lectin adhesion molecules that bind to ligands which are decorated with fucosylated glycostructures similar or identical to sialyl Lewis X \(^3,4\). LAD II patients display a generalized defect in fucosylation that affects the selectin ligands, strongly impairing leukocyte – endothelial cell interactions. This causes reduced extravasation of neutrophils and immunodeficiency. The fucosylation defect also leads to prominent neutrophilia as well as mental and growth retardation \(^5-8\).

We and others have identified mutations in the gene that codes for the GDP-fucose transporter (GFTP) as the genetic defect causing the disease \(^9,10\). The GFTP resides in the Golgi membrane where it serves to transport the nucleotide sugar GDP-fucose into the Golgi lumen \(^9,11\). GDP-fucose is synthesized in the cytosol mainly from mannose and glucose via a de-novo pathway and, to a minor extent, via a salvage pathway that uses either exogenous L-fucose or fucose derived from degraded glycoconjugates \(^12,13\). Following transport into the Golgi by the GFTP the nucleotide sugar serves as a substrate for fucosylation reactions carried out by several fucosyltransferases. The human GFTP is a protein of 364 amino acids with ten transmembrane (TM) domains and the amino and carboxy termini exposed to the cytosol \(^9,10\) and Figure 2). The protein is expressed as a homodimer and translocates GDP-fucose into the Golgi lumen in exchange for GMP.

So far, six LAD II patients have been identified of whom five were analysed for the genetic defect. These patients all show mutations in the GFTP gene. The patient in whom we previously identified the genetic cause of LAD II is of Turkish origin and displays a single
amino acid exchange (R147C) in the fourth TM domain of the GFTP due to a point mutation in the gene \(^9\). Out of four Israeli patients of Arab origin three were analysed for the genetic defect and were found to display a different single amino acid exchange (T308R) in the ninth TM domain \(^{10,14}\). Recently, the sixth LAD II patient who is of Brazilian origin was described \(^{15}\). The GFTP gene of this patient showed a nucleotide deletion (ΔG588) causing a reading frame shift that leads to a premature termination codon. The resulting protein was predicted to be severely truncated lacking the last five TM domains and the cytoplasmic C-terminus.

A therapy for LAD II was established when we orally administered L-fucose to the Turkish patient and found that this treatment caused reappearance of functional selectin ligands and a complete abrogation of the patient’s immunodeficiency and neutrophilia \(^{16,17}\). Using identical or similar protocols three more LAD II patients were treated with varying success. Whereas fucose supplementation apparently had no effect on two of the Israeli patients \(^{18,19}\) the therapy caused the same beneficial effects in the Brazilian patient as in the Turkish patient, albeit accompanied by a controllable autoimmune reaction against refucosylated antigens \(^{15}\). How GDP-fucose is transported into the Golgi in LAD II patients under conditions of therapy is not understood.

The mechanisms by which defective GFTPs cause reduced or absent transport of GDP-fucose in LAD II patients are ill-defined. In particular, it is not established if and where the defective transport molecules are expressed. In this respect three alternatives are possible: The defective GFTP is expressed and resides in the Golgi but is deficient in transport function due to its mutation. Second, the transporter may be expressed, but not in the Golgi, or third, the mutated protein may not be expressed at all. So far, the expression and localisation of the GFTP has only been analysed for the Turkish patient. We found that the defective GFTP of this patient is correctly localized in the Golgi but non-functional \(^8\).

Here we analyse a new LAD II patient and find that her GFTP is retained in the endoplasmic reticulum (ER) due to a missing TM domain. In addition, this patient’s GFTP
lacks a region in the C-terminal cytoplasmic domain rendering the protein inactive. We show that all other LAD II patients can be subdivided by the two mechanisms which we detected in the Turkish and the new LAD II patient, respectively. Interestingly, despite the dual defect of the GFTP, fucosylation of cells of the new LAD II patient can be restored by exogenous fucose indicating that fucose supplementation therapy of LAD II is based on an alternative GDP-fucose transport mechanism.
Methods

Patient

Patient IS was born at term. At birth, she was in good condition and showed no obvious dysmorphism. Her birth weight was 2.81 kg (9th – 25th centile) and her occipitofrontal circumference (OFC) was 33.8 cm (25th – 50th centile). She remained in the maternity hospital for a week because of slow feeding. At four months of age she was referred to hospital because of poor feeding, failure to thrive and hypotonia. Her head circumference was noted to have fallen to 3rd centile and weight was below 3rd centile. She was also noted to have spiky, brittle hair, frontal bossing, hypertelorism, a cardiac murmur, and short proximal limb segments. She has had few significant infections, although she had widely disseminated infection of the skin with Herpes simplex virus at the age of four, which required hospital admission. She had delayed eruption of teeth and has mild periodontitis. She had an episode of pustular dermatitis of her hands and feet at the age of one. She is allergic to egg. At the age of 2 years she was diagnosed with epileptic seizures and treated with sodium valproate. Blood levels of factor XI, protein C, free and total protein S and antithrombin activity are normal.

Her main problems have been poor weight gain and global developmental delay. She required overnight nasogastric feeds for a period of time at the age of 2 years. She has continued to have poor oral intake and a gastrostomy tube is being considered. She is significantly delayed in her development. Formal assessment at the age of 3 ½ years showed her to be performing at 12-15 month level in most areas with locomotor skills scoring slightly higher than visual/cognitive areas. She speaks two words, communicating her needs only by vowel sounds and cries. A magnetic resonance imaging (MRI) brain scan at 4 months of age was normal, with no evidence of a neuronal migration disorder or other structural abnormality. She suffers from bilateral myopic astigmatism. Blood samples, cultured fibroblasts and DNA were used according to institutional guidelines of the Yorkhill NHS
Trust, Royal Hospital for Sick Children, Glasgow, UK, and written informed consent of the patient's parents was provided according to the Declaration of Helsinki.

**Cells**

Fibroblasts derived from LAD II patient IS, the Turkish LAD II patient \(^7,9\) and healthy individuals as well as COS-7 cells were cultured at 37°C with 5% CO\(_2\) in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) containing 15% (fibroblasts) or 10% (COS-7) fetal calf serum (PAN Biotech, Aidenbach, Germany). Peripheral blood neutrophils were obtained from anticoagulated blood in a Histopaque 1077 / Histopaque 1119 double gradient (Sigma-Aldrich, Deisenhofen, Germany) and used for flow cytometry after 2 washes with PBS / 0.5% BSA. Erythrocytes were obtained from the pellet of the double gradient.

**Sequence analysis and DNA cloning**

Genomic DNA was prepared from whole blood using a kit (Qiagen, Hilden, Germany). Both exons of the GFTP gene (AF323970) were sequenced employing standard procedures \(^20\) using the following primers (in 5’-3’ direction):

- TGGACTCCAGGGAATCAGAGTTC
- ACTGCTTCAGTCCCCATGACC
- CTTCTTCCTCCTCGTCCTCATCC
- ACCGCCTTCCTACTGTGCTGG

The PCR-products were purified and sequenced using an automated DNA sequencer (Applied Biosystems, Weiterstadt, Germany).

Cloning of GFTP cDNAs without GFP tag: The GFTP cDNA from patient IS was cloned by RT-PCR (2 independent RT reactions) using MMuLV reverse transcriptase (Stratagene, La Jolla, CA) and total RNA isolated with the RNeasy Mini Kit (Qiagen) from the patient’s fibroblasts. RT-PCR reactions were performed with the following primers (sense
primer followed by antisense primer, in 5’-3’ direction):
CCACCATGGCGCTGACCGGGGCCTCAGACCCCTC, TCACACCCCCATGGCGCT.

For sequencing and some of the complementation studies the PCR product (with a stop codon at the 3’ end, i.e. expressed without GFP-tag) was cloned into pcDNA3.1/CT-GFP TOPO (Invitrogen). Sequencing was done using the Big Dye 3.1 sequencing kit (Applied Biosystems, Foster City, CA). GFTP cDNAs (without tag) from healthy donors and the Turkish LAD II patient in pcDNA3.1/CT-GFP TOPO were published before 9.

Cloning of GFTP cDNAs with N-terminal GFP tag: For targeting and complementation experiments the GFTP cDNAs from patient IS, the Turkish patient and a healthy donor in pcDNA3.1/CT-GFP TOPO were used in PCR reactions to create fusion proteins with a N-terminal GFP tag. For the deletion constructs Δ25C and Δ22C the GFTP cDNA of a healthy donor was used as a template. In the PCR reactions the sense primer ATGGCGCTGACCGGGGCCTCAGACCCCT was used. Antisense primers were: for full-length constructs from patients and healthy control: TCACACCCCCATGGCGCT; for construct Δ25C: TCACCAGGTGTAGGCGGAGGAG; for construct Δ22C: TCAGCCCCTGACCACCCAGGTGA. The PCR products were cloned into pcDNA3.1/NT-GFP TOPO (Invitrogen) and sequenced.

Cloning of GFTP cDNAs with C-terminal GFP tag: To create deletion construct Δ37N with a C-terminal GFP tag a PCR was performed using the GFTP cDNA (without tag) from a healthy donor in pcDNA3.1/CT-GFP TOPO as template using sense primer CCACCATGGCGATTCAGATCGCGCTGT and antisense primer GCACCCCCATGGGCTT. The PCR product was cloned into pcDNA3.1/CT-GFP TOPO (Invitrogen) and sequenced. C-terminally tagged GFTPs from the Turkish patient and healthy donors were described before 8,9.

Site-directed mutagenesis
GFTPs bearing the nucleotide deletion ΔG588 of the Brazilian LAD II patient \(^{15}\) and the C923G transversion of LAD II patients of Arab origin \(^{10,14}\) were generated by site-directed mutagenesis. Using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) PCR reactions were performed according to the manufacturer’s protocols with wild-type GFTP cDNA in pcDNA3.1/NT-GFP TOPO (see above) as template and the following primers (sense, antisense): for introduction of the ΔG588 deletion: 

\[
\text{GGCACCCTGTGCTGGGCACCGTCTTC,}
\]

\[
\text{GAAGACGGTGCCCAGCACGACAGGGTGCC;}
\]

for introduction of the C923G transversion: 

\[
\text{CCAAAGGCTGTGCCCAGAGAGTGCTGGCCGTCTC,}
\]

\[
\text{GAGCACGGCCAGCAGCTCTCTGGGCACAGGCCTTGG.}
\]

For each mutated GFTP PCR products from two independent PCR reactions were produced, sequenced and used in transfection experiments.

**Flow cytometry**

Flow cytometry was performed according to standard protocols \(^{16}\). Biotinylated *Aleuria aurantia* lectin (AAL, Vector laboratories, Burlingame, CA), anti-sLe\(^x\) antibody CSLEX-1 (ATCC, Manassas, VA), anti-Le\(^x\) antibody (BD Pharmingen, Heidelberg, Germany), anti-H-antigen antibody (Mast Diagnostica, Reinfeld, Germany) and negative control IgM mAb (BD Pharmingen) were used at 10 µg/ml. AAL was detected with phycoerythrin-conjugated streptavidin. Streptavidin-PE alone served as negative control. E- and P-selectin-IgG and VE-cadherin-IgG constructs were published before \(^{21,22}\).

**Complementation and targeting experiments**

LAD II fibroblasts and COS-7 cells were seeded on slides in 90-mm dishes, transfected with 4 µg of the indicated DNAs using the GeneJammer transfection reagent (Stratagene) as described \(^9\) and cultured for 48 h (in some experiments in the presence of 0.03
- 10 mM L-fucose (Sigma-Aldrich). Subsequently, the cells were fixed and permeabilized as described and stained. Biotin-conjugated *Aleuria aurantia* lectin (10 μg/ml) (Vector laboratories) and Streptavidin-Cy3 (Jackson Immunoresearch, West Grove, PA) were used for specific detection of α1,3- and α1,6-linked fucose. GFP was detected with a polyclonal anti-GFP antibody (1:500, Abcam, Cambridge, UK) and Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes-Invitrogen, Karlsruhe, Germany). The Golgi was stained with antigolgin-97 antibody (mouse IgG, Molecular Probes-Invitrogen) and Alexa Fluor 568-conjugated anti-mouse IgG (Molecular Probes-Invitrogen). For detection of the endoplasmic reticulum COS-7 cells were co-transfected with 2 μg of the subcellular localisation vector pDsRed-ER (red autofluorescence) (BD Pharmingen) and 3 μg of the various GFTP cDNAs. For analysis a 20x objective on a Zeiss Axioscope 50 microscope (Zeiss, Oberkochen, Germany) and a SPOT imaging system (Diagnostic Instruments, Sterling Heights, MI) was used except for cells transfected with pDsRed-ER which were analysed with a 100x oil immersion objective. For complementation experiments that were analysed by flow cytometry, LAD II fibroblasts were transfected by nucleofection using the “Human Dermal Fibroblast Nucleofactor” kit (Amaxa Biosystems, Cologne, Germany) and a Nucleofactor I transfection device (Amaxa Biosystems) according to the manufacturer’s neonatal fibroblast transfection instructions and grown for 48 h in FGM-2 Bulletkit medium (Cambrex BioScience).
Results

Clinical presentation of a new LAD II patient

The girl (named patient IS in this report) is the child of parents who are British Asians of Pakistani origin and are first cousins. The patient suffers from global developmental delay and poor weight gain combined with poor oral intake. The patient was noted to have short proximal limb segments, hypotonia, microcephaly, a depressed nasal bridge, hypertelorism and bilateral myopic astigmatism (Fig. 1A). At the age of two the girl developed epileptic seizures and was treated with sodium valproate. In addition, the patient shows a significant delay in psychomotor development (see Methods). She has had few significant infections including pustular dermatitis of her hands and feet and widely disseminated Herpes simplex virus infection of the skin. The patient had delayed eruption of teeth and has mild periodontitis. Total white blood cell counts as well as neutrophil counts were constantly elevated in the patient (Fig. 1B).

Neutrophils of the new LAD II patient show strong hypofucosylation and fail to express selectin ligands

We analysed the fucosylation of the new patient’s neutrophils using the *Aleuria aurantia* lectin (AAL) which is specific for α1,3- and α1,6-fucosylated glycoconjugates. We found that binding of this lectin to patient cells was strongly reduced with a residual staining of only 1.5% of healthy levels indicating a strong hypofucosylation (Figure 1C). AAL-binding to the patient’s erythrocytes was equivalently reduced and was accompanied by a lack of A- and B-blood group antigens including the fucosylated blood group precursor, the H-antigen (not shown). Moreover, the fucosylated Lewis X antigen (Le^a^) was absent from the patient’s neutrophils as was its sialylated form, sialyl Lewis X (sLe^a^), which is the prototype glycostructure that is required for binding of the selectins (Figure 1C). Absence of binding of
E-selectin- and P-selectin-IgG chimeric proteins to the patient’s neutrophils showed that the hypofucosylation in the patient leads to a defect in expression of functional selectin ligands (Figure 1).

Taken together, the clinical and molecular data show that patient IS suffers from a strong hypofucosylation that causes typical LAD II manifestations. Thus, patient IS is the 7th LAD II patient that has been reported by now.

**The GFTP gene of the new LAD II patient displays a premature translational termination codon**

Analysis of the genomic sequence of the patient’s GDP-fucose transporter gene revealed a homozygous G969A transition in exon 2 resulting in a premature TGA termination codon in the reading frame. Sequencing of cDNAs of the respective transcripts revealed the same mutation (Figure 2A). Both parents and an apparently healthy sibling of the patient were heterozygous for the mutation. Due to the premature termination codon the patient’s GFTP molecule is predicted to be truncated after amino acid L322 and to lack the last of its ten transmembrane (TM) domains as well as the C-terminal cytoplasmic domain (Figure 2B).

**The GFTP of the new LAD II patient is non-functional**

The function of the new patient's GFTP was tested in transfection experiments in comparison to the wild-type GFTP and the GFTP of the Turkish LAD II patient bearing the single amino acid substitution R147C. In order to detect expression of the transporters they were N-terminally tagged with GFP. The molecules were expressed in LAD II fibroblasts derived from patient IS which show strong hypofucosylation as detected by lack of AAL binding (Figure 3A). We found that transient transfection of the wild-type transporter into these LAD II cells restored fucosylation in all cells which expressed the GFP-tagged molecule (Figure 3B). In contrast, neither the GFTP of the Turkish patient nor the GFTP of patient IS were able
to restore fucosylation in the fibroblasts showing that the GFTP of the new LAD II patient is non-functional (Figure 3B). Identical results were obtained when fibroblasts of the Turkish patient were transfected with the GFTPs (not shown). The functional defect of the new patient’s GFTP was also seen in complementation experiments with untagged molecules (data not shown).

**The GFTP of the new LAD II patient fails to localize to the Golgi**

The GDP-fucose transporter of healthy individuals resides in the Golgi membrane (9,11 and Figure 4) where it serves to provide GDP-fucose for the action of several fucosyltransferases. We have previously noted that the defective GFTP of the Turkish patient with the R147C amino acid exchange was correctly expressed in the Golgi as shown by colocalisation with a Golgi marker (8 and Figure 4). We now studied the subcellular distribution of another defective GFTP that was found in three LAD II patients of Arab origin and bears a different single amino acid exchange (T308R) 10,14. We found that the defective GFTP of these patients was also correctly located in the Golgi (Figure 4). However, in contrast to these defective transporters bearing single amino acid exchanges the truncated GFTP of patient IS did not localize to the Golgi. Instead, we found the molecule to be distributed virtually over the entire cell body (Figure 4). This was not the consequence of overexpression of the defective GFTP since i) in the same transfection experiments the wild-type GFTP was well confined to the Golgi, ii) we employed a transfection method that resulted in very low autofluorescence of the GFP so that anti-GFP antibodies had to be used for detection and iii) because cells displaying even very faint staining with anti-GFP showed the same staining pattern (not shown). We conclude that the new patient's GFTP has lost its ability to localize to the Golgi, the compartment in which its function is required.

We suspected a localisation of the new patient’s GFTP in the endoplasmic reticulum (ER). We therefore used COS-7 cells in which the reticular staining pattern of the ER
including the nuclear envelope is more clearly visible than in fibroblasts and co-transfected them with GFP-tagged GFTPs and a vector coding for a fluorescent protein that localizes to the ER due to a “KDEL” ER retention signal. We found that neither GFP alone nor the GFP-tagged wild-type GFTP nor the GFTP of the Turkish patient colocalized with the ER marker (Figure 5). In contrast, the transporter of patient IS gave a GFP label that considerably overlapped with the ER marker (Figure 5). Localisation of the patient’s GFTP in the ER could also be observed in fibroblasts although in these cells the ER was more difficult to distinguish from the cytoplasm than in COS-7 cells (not shown). These data suggest that the truncated GFTP is not able to leave the ER in order to enter the Golgi.

Recently, Hidalgo et al. described a LAD II patient of Brazilian origin who expresses a non-functional GFTP with an even more severe truncation. This defective protein was predicted to lack the C-terminal five TM domains plus the C-terminal cytoplasmic domain due to a frame shift in its gene. The subcellular localisation of this GFTP had not been determined. Since the deletion in this GFTP includes the deletion in patient IS we expected the GFTP of the Brazilian patient to be unable to localize to the Golgi. Indeed, when a tagged GFTP with the mutation of the Brazilian patient was expressed in COS-7 cells it did not localize to the Golgi but was mainly found in the ER resembling the subcellular localisation of the GFTP of patient IS (Figure 5). Thus, in both known LAD II patients with truncated GFTPs the defective molecule is mislocalized.

**Absence of the last transmembrane domain causes mislocalisation of the GFTP**

We next investigated which of the lacking regions in the GFTP of patient IS, the 10th TM domain or the cytoplasmic domain, is responsible for the mislocalisation and defective function of the transporter. We first asked whether reconstituting the 10th TM domain while still omitting the cytoplasmic C-terminus would result in an active GFTP. We predicted the 10th TM domain of the GFTP to end with amino acid A336 using two structure prediction
programs. In order to compensate for uncertainties which are immanent to such predictions we generated a C-terminal deletion construct (Δ25C) which terminates after amino acid W339, making sure that the entire 10th TM domain was included. We found that this construct was correctly located in the Golgi (Figure 6A). This shows that the lack of the 10th TM domain in the GFTP of patient IS is responsible for the mislocalisation of the transport molecule. This result also implies that the lack of the last TM domain in the Brazilian patient’s GFTP is sufficient to cause its retention in the ER.

**Absence of a membrane-proximal C-terminal cytoplasmic region renders the GFTP inactive**

Interestingly, complementation of LAD II fibroblasts with construct Δ25C did not restore fucosylation (Figure 6B). This shows that Golgi localisation of Δ25C was not sufficient to make this truncated construct functional and that a region within the C-terminal cytoplasmic domain is required for transport function. We next sought to identify this functionally important cytoplasmic region. We found that an elongation of construct Δ25C by only three more amino acids resulted in a transporter molecule (construct Δ22C) that did not only localize to the Golgi but also restored AAL staining in all transfected LAD II fibroblasts in a manner indistinguishable from the wild-type GFTP (Figure 6A and B). Cell surface AAL staining below saturation was also compared and quantified and was found to be very similar following transfection with wild-type GFTP and construct Δ22C, respectively (Supplemental Figure 1). This shows that one or more of the membrane-proximal cytoplasmic amino acids 340-342 (VRG) are required for the function of the GDP-fucose transporter molecule. Interestingly, in contrast to the C-terminal domain described above, deletion of the entire N-terminal cytoplasmic domain in construct Δ37N neither influenced the subcellular localisation nor the function of the GFTP (Figure 6A and B).
Thus, the known LAD II patients with truncated GFTPs suffer from a dual defect of the transport molecule, a deficiency in Golgi localisation due to the lack of one or more TM domains, and an additional defect in transport function due to the absence of a functionally important region within the C-terminal cytoplasmic domain.

**Effect of exogenous fucose on the fucosylation of the new patient’s fibroblasts**

It is still an open question whether the successful fucose-based therapy of LAD II patients that we first tested in the Turkish patient \(^{16,17}\) is based on a residual function of the GFTP or on another transport system that allows GDP-fucose transport into the Golgi as soon as exogenous fucose is given. We tested the effect of culturing fibroblasts of patient IS in the presence of L-fucose and found that the fucose supplementation with 0.1 – 10 mM L-fucose caused refucosylation of the cells (Figure 7A and Supplemental Figure 2) while it did not alter the aberrant localisation of the transport molecule (Figure 7B). This is in line with results obtained with the Brazilian patient who was successfully treated with L-fucose \(^{15}\) despite the mislocalisation of his GFTP (Figure 5).

Our stainings do not fully exclude that a small percentage of the truncated GFTPs is localized in the Golgi. We therefore tested the function of the truncated GFTP of patient IS under conditions of fucose supplementation. We transfected LAD II fibroblasts with the wild-type GFTP and the truncated GFTP of patient IS, respectively, and cultured the cells in medium containing a suboptimal concentration of L-fucose at which the corrective transport system is active causing a restricted refucosylation. Under these conditions the overexpression of a transporter that is active during fucose supplementation is expected to further increase fucosylation. We find that whereas the wild-type GFTP is able to perform in such a way the GFTP of patient IS remains inactive (Supplemental Figure 3). Even construct Δ25C which fully locates to the Golgi but lacks amino acids 340-342 is unable to induce fucosylation under these conditions (Supplemental Figure 3). This shows that even if some of the truncated
GFTP molecules are located in the Golgi they cannot account for the therapeutic effect of exogenous L-fucose.

Since it is difficult to explain how a non-functional transport molecule that, in addition, is not able to localize to the Golgi can support fucosylation events in the Golgi, these data strongly support -although they do not formally prove- the notion that an alternative transport system is responsible for the successful treatment of LAD II patients with L-fucose.
Discussion

In this report we define a group of LAD II patients who display a novel dual defect of the GFTP including, first, truncations of one or more transmembrane domains causing mislocalisation of the transport protein and, second, the absence of a region in the C-terminal cytoplasmic domain which is required for the function of the transporter. In addition we show that exogenous fucose restores fucosylation in cells of such a patient which suggests the presence of an alternative GDP-fucose transport system.

The mislocalized truncated GDP-fucose transporters, when ectopically expressed, are mainly located in the ER presumably because their truncation leads to recognition by the ER-resident protein quality control system similarly to truncated CMP-sialic acid transporters that have recently been detected in a sialylation-deficient patient. Expression of the GFTP, however, is required in the Golgi because the fucosyltransferases that catalyse fucosylation of N- and O-linked carbohydrate chains reside in that compartment. Thus, it is very likely that the mislocalisation of truncated GFTPs contributes to the fucosylation defect in the affected LAD II patients.

Staining with ER markers (e.g. Figure 5) visualize a very dense network that makes it difficult to tell whether an ER-resident protein is entirely excluded from other compartments like the Golgi. We therefore cannot exclude from these stainings that a small percentage of the truncated GFTPs may still be targeted to the Golgi. However, our double stainings for GFP-tagged TPs and the Golgi marker golgin-97 show no colocalization (Figure 4). In addition, even if some of the truncated GFTP was expressed in the Golgi the transporter would not be functional due to the lack of one or more of the C-terminal amino acids V340-R341-G342. Thus, the LAD II patients with truncated GFTPs suffer from a dual defect of mislocalisation and non-function of the transport molecule.
We have previously noted that two splicing variants of the human GFTP exist, one which is full-length, and another in which the first 13 amino acids are missing. We and others showed that mRNAs for both variants are expressed and that the corresponding proteins are fully functional \(^9,^{10}\). Now we show that the entire N-terminal cytoplasmic domain of 24 amino acids is dispensable for Golgi localisation and function. More interestingly, the cytoplasmic C-terminus bears a stretch of three amino acids which appears to be required for transport function of the GFTP. Data on the role of the C-terminal cytoplasmic domain in other NSTs are inconsistent. Deletion of this domain in the mouse UDP-galactose transporter resulted in a construct that could not be expressed \(^{25}\) whereas replacement of the C-terminal cytoplasmic domain of the human UDP-galactose transporter by the corresponding domain of the human CMP-sialic acid transporter had no effect \(^{26,27}\). Deletion of the C-terminal cytoplasmic domain of the yeast GDP-mannose transporter was reported to cause loss of function \(^{28}\). However, in another study these data could not be reproduced \(^{29}\). Future studies will have to address the exact role of the three cytoplasmic amino acids 340-342 in the GFTP, whether it be to support the assembly of the transporter protein dimer or the binding and translocation of the nucleotide sugar.

Several years ago we treated the first LAD II patient (the patient of Turkish origin) with oral L-fucose and found that this treatment induced refucosylation of glycostructures including selectin ligands and that it cured the patient’s immunodeficiency \(^{16,17}\). This child is still immunologically healthy after six years of continuing treatment (own unpublished results). It is clear that the salvage pathway is responsible for the cytoplasmic biosynthesis of GDP-fucose from exogenous L-fucose. However, it is not known how sufficient amounts of GDP-fucose are transported into the Golgi under conditions of therapy. It is possible that with elevated GDP-fucose levels in the cytoplasm the defective GFTP in the Turkish patient has residual transport activity or that an alternative unknown transport molecule translocates GDP-fucose into the Golgi.
The fucose-based therapy was repeated in two Arab LAD II patients without success \cite{18,19}. One of these patients was analysed for the genetic defect and was found to bear the T308R mutation \cite{14}. Since this mutation differed from that in the Turkish patient (R147C) it was assumed that the different outcome of the therapy was due to the different GFTP mutations in the patients \cite{14}. This notion implied that an alternative GDP-fucose transport mechanism is not relevant and was underscored by studies on one Arab patient whose Golgi vesicles were shown to display a decreased maximal rate of transport \cite{19} whereas this feature was not seen in the Turkish patient \cite{30}.

In contradiction to the notion mentioned above we now show that LAD II cells can be refucosylated with exogenous fucose even if the GFTP is mislocalized and remains mistargeted during fucose supplementation. In addition, we show that the severely truncated GFTP of the Brazilian patient who was successfully treated with oral L-fucose \cite{15} is also mislocalized. Our immunofluorescence stainings do not fully exclude that a small percentage of the truncated GFTPs is localized in the Golgi. However, it is very unlikely that this can account for the refucosylation by exogenous L-fucose for the following reasons: a) Even if some of the truncated GFTPs are located in the Golgi the lack of amino acids 340-342 would keep them inactivated. b) Overexpression of the truncated GFTP of patient IS and of the Golgi-located construct Δ25C under conditions of suboptimal supplementation with L-fucose did not further increase fucosylation.

These findings are difficult to reconcile with the notion that the defective GFTP itself is responsible for the therapeutic effect of fucose supplementation and rather strongly support the assumption that an alternative transport system is responsible for the success of oral L-fucose treatment.

The nature of the putative alternative transport system is unknown. No other transport molecule that is able to transport GDP-fucose has been described and data base searches reveal no protein with high homology to the GFTP (own unpublished results). Recently, it
was found that O-fucosylation, a process that directly attaches fucose to the protein core and which requires GDP-fucose, takes place in the endoplasmic reticulum. Thus, an ER-based GDP-fucose transport system can be postulated which appears to be active in at least one LAD II patient since no defect of O-fucosylation of notch could be detected in this individual. Chen et al. recently suggested that a multi-transmembrane protein termed SLC35C2 (gene CGI-15 / Ovcov1) might transport GDP-fucose into the ER but expression of this protein reduced the Golgi-based fucosylation of LeX arguing against SLC35C2 to be the alternative transporter. Thus, the transporter which is active during fucose supplementation remains elusive. We speculate that one of the remaining genes of the SLC35 family coding for nucleotide sugar transporters of unknown function is responsible for the therapeutic effect of L-fucose.

In conclusion, this study identifies a dual defect in subcellular localisation and function by which a truncated GFTP causes LAD II and strongly supports the notion that the fucose-based therapy of LAD II is dependent on an alternative GDP-fucose transport system.
Acknowledgments:

We thank Carlos B. Hirschberg (Boston University) and Andrés Hidalgo (Mount Sinai School of Medicine, New York) for helpful discussions. We thank Ute Ipe for excellent technical assistance.
References:


Figures:

Figure 1: Clinical phenotype, blood cell counts and fucosylation in neutrophils of patient IS. (A) Patient IS at the age of 26 months. A broad and depressed nasal bridge and divergent strabism are present. Gastric tube feeding is necessary. (B) Peripheral total white blood cell (WBC) and neutrophil counts of the patient during the first 2 years of life. Total WBC counts were permanently elevated. (C) Fucosylation and selectin ligand expression in neutrophils of the patient. Healthy and patient neutrophils were incubated with the fucose-specific *Aleuria aurantia* lectin (AAL), anti-Le^x^- or anti-sLe^x^-antibodies, and selectin-IgG (Sel-IgG) chimeric proteins, respectively, before they were analysed by flow cytometry. Negative controls were secondary antibody only (for AAL), control IgM antibody (for anti-Le^x^- and anti-sLe^x^-antibodies) and VE-cadherin-IgG (for selectin-IgG), respectively.

Figure 2: LAD II patient IS displays a premature translational termination codon in the GFTP gene. (A) The genomic sequence coding for the GFTP and the respective transcripts obtained from two independent RT-PCR reactions were sequenced and found to bear a G969A nucleotide substitution resulting in a premature stop codon. (B) Predicted topology of wild-type and mutated GFTP. Boxes represent transmembrane domains. The GFTP of LAD II patient IS is predicted to be truncated after the ninth transmembrane domain.
**Figure 3: The GFTP of the new LAD II patient is non-functional.** (A) Fibroblasts derived from a healthy control donor and from patient IS were stained with AAL. Perinuclear fucosylation in the Golgi is seen in healthy but not in patient cells. (B) Complementation of LAD II fibroblasts with green fluorescent protein (GFP)-tagged GFTPs of a healthy donor (wild-type), the Turkish LAD II patient and patient IS, respectively. Cells were double-stained with anti-GFP and AAL. One representative experiment out of six is shown. Bars, 10 µm.

**Figure 4: The GFTP of patient IS does not localize to the Golgi.** Fibroblasts derived from LAD II patient IS were transiently transfected with GFP-tagged GFTPs from a healthy donor, the Turkish LAD II patient, three Arab LAD II patients and patient IS, respectively. Cells were double-stained with anti-GFP antibodies and antibodies against the Golgi protein golgin-97. One representative experiment out of three is shown. Bar, 10 µm.

**Figure 5: The truncated GFTPs of two LAD II patients localize preferentially to the ER.** COS-7 cells were transfected with GFP-tagged GFTPs of a healthy individual and of the indicated LAD II patients together with vector pDsRed-ER coding for an ER-resident fluorescent protein. Cells were stained with anti-GFP antibodies. One representative experiment out of three is shown. Bar, 10 µm.

**Figure 6: Two missing regions in the patient’s GFTP are required for Golgi localisation and transport function.** The C-terminal deletion mutants Δ25C and Δ22C lack the last 25 and 22 amino acids, respectively. These constructs bear an N-terminal GFP-tag. The N-terminal deletion mutant Δ37N lacks the first 37 amino acids and bears a C-terminal GFP-tag. Only one of ten transmembrane domains is shown. Fibroblasts of patient IS were transfected with the deletion constructs and analysed for the GFP-tag and golgin-97 (A) or GFP and AAL (B). Construct Δ37N was compared with a C-terminally tagged wild-type GFTP that was
functional and located in the Golgi (not shown). One representative experiment out of three is shown. Bars, 10 µm.

**Figure 7: Exogenous fucose can restore fucosylation in cells of the new patient.** (A) Fibroblasts derived from patient IS were cultured without or with 10 mM L-fucose for 48 h before they were analysed for AAL-binding. (B) Fibroblasts of patient IS were transfected with the GFP-tagged GFTP of this patient and cultured without or with 10 mM L-fucose for 48 h. Staining of GFP and golgin-97 is shown. One representative experiment out of three is shown. Bars, 10 µm.
Figure 1A
Figure 1B
Figure 1C
Figure 2

A

wt
patient IS

wt
patient IS

...AGCTTCCTCTGGTGGACG...
...AGCTTCCTCTGATGGACG...

S F L W W T
S F L

322 Stop

B

wild-type GFTP:

Golgi lumen

N

cytosol

58 75 135 142 185 197 250 265 311 320
1 2 3 4 5 6 7 8 9 10
41 92 118 160 168 214 233 282 294 336

C 364

GFTP of patient IS:

C 322

N

58 75 135 142 185 197 250 265 311
1 2 3 4 5 6 7 8 9
41 92 118 160 168 214 233 282 294
Figure 3

A

control fibroblasts

fibroblasts of patient IS

B

wild-type GFP

GFP of Turkish patient

GFP of patient IS
Figure 4

golgin-97  GFP  merge

wild-type GFTP

GFTP of Turkish patient

GFTP of Arab patients

GFTP of patient IS
Figure 5

- ER marker
- GFP
- merge

GFP alone

wild-type GFTP

GFTP of Turkish patient

GFTP of patient IS

GFTP of Brazilian patient
Figure 6A
Figure 6B

B

Δ25C GFP

Δ22C GFP

Δ37N GFP

GFP

AAL

phase contrast

320
10
336
C

320
10
336
C

58
1
41
N
Figure 7

A

without L-fucose  with L-fucose

AAL

B

golgin-97 (red)  GFP (green)

From www.bloodjournal.org by guest on August 16, 2017. For personal use only.
Leukocyte adhesion deficiency II patients with a dual defect of the GDP-fucose transporter

Yvonne Helmus, Jonas Denecke, Sviatlana Yakubenia, Peter Robinson, Kerstin Luhn, Diana L Watson, Paraic J McGrogan, Dietmar Vestweber, Thorsten Marquardt and Martin K Wild