Complementation cloning identifies CDG-IIc, a new type of congenital disorders of glycosylation, as a GDP-fucose transporter deficiency

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Congenital disorders of glycosylation (CDG) comprise a rapidly growing group of inherited disorders in which glycosylation of glycoproteins is defective due to mutations in genes required for the assembly of lipid-linked oligosaccharides, their transfer to nascent glycoproteins (CDG-I) or the processing of protein-bound glycans^{1,2} (CDG-II). Previously, a defect in the GDP-fucose import into the lumen of the Golgi was identified in a person with CDG (A.C.) with a general deficiency of fucosyl residues in glycoproteins³. This patient presents the clinical features of leukocyte adhesion deficiency type II (LAD II) including mental retardation, short stature, facial stigmata, and recurrent bacterial peripheral infections with persistently elevated peripheral leukocytes⁴⁻⁷. Using a fucose-specific, lectin-staining procedure for detection of fucosylated glycoproteins and a retroviral cDNA library, we isolated a cDNA complementing the fucosylation defect in the patient's fibroblasts. The cDNA encodes a highly hydrophobic protein of 364 amino acids with multiple putative transmembrane domains. Restoration of GDP-fucose import activity in Golgi-enriched vesicles from the patient's fibroblasts verified the GDP-fucose transporter activity of this protein. We identified two missense mutations in the GDP-fucose transporter cDNA of patient A.C. and of two other people with LAD II. Thus complementation cloning allowed us to identify the human GDP-fucose transporter cDNA and GDP-fucose transporter deficiency as a cause for a new type of CDG. Following the recent recommendations^{2,8} for the nomenclature for CDG, this new type is classified as CDG-IIc (formerly LAD II).

We used a functional cloning approach to isolate a cDNA that complements the phenotype of the patient's fibroblasts. We split a human liver cDNA library in a *MMLV*-based retroviral vector into 48 A-pools, each representing 2,500 independent clones. Each cDNA pool was packaged into infectious viral particles using ecotropic and amphotropic packaging cell lines. After repeated infection of patient fibroblasts and culturing for 7 days, the fibroblasts were fixed and reacted with biotinylated *Aleuria aurantia* lectin (AAL), which is specific for α -1,6- and α -1,3-linked fucosyl residues. The AAL staining of control and patient fibroblasts is shown (Fig. 1a,b), as well as the restoration of the fucosylation defect in patient fibroblasts infected with pool A44 (Fig. 1c). We divided pool A44 into 48 B-pools, each representing 200 independent clones. Screening of the B-pools revealed the presence of complementary cDNA in pool B23. In

the following screening round, we split pool B23 into 25 C-pools, each representing 20 independent clones. Pool C23 contained the complementing cDNA. Individual clones of pool C23 were amplified and screened for complementation in round D. Clone D5 restored fucosylation in 15–50% of the patient fibroblasts (Figs. 1d and 5a). The GDP-fucose import into Golgi-enriched microsomes of patient fibroblasts was restored to 63% and 163% of that in controls depending on the percentage of infected cells (Fig. 2). In patient fibroblasts selected for stable expression of D5 (Fig. 5c), GDP-fucose import was 315% of that in control fibroblasts (Fig. 2). The restoration of glycoprotein fucosylation and of GDP-fucose import into Golgi-enriched microsomes established that the cDNA insert of clone D5 encodes a GDP-fucose transporter.

The cDNA insert is approximately 3.1 kb and contains an ORF of 1,095 bp preceded by 609 bp of 5' untranslated sequence. A BLAST search showed identity with several ESTs and with the human placenta mRNA NM_018389, of unknown function. The sequence of D5 and NM_018389 diverge at the 5' end. NM_018389 contains a 153-bp 5' UTR followed by an ORF of only 1,056 bp lacking the information for the 13 amino-terminal amino acids encoded by D5 (Web Fig. A). Sequence comparison of the D5 cDNA identified putative orthologs in *Sus scrofa*, *Bos*

Fig. 1 AAL staining of control and patient fibroblasts. Control fibroblasts (a), noninfected patient fibroblasts (b), and patient fibroblasts transiently expressing the cDNA from pool A44 (c) or the cDNA from pool D5 (d) were histochemically labeled with biotinylated AAL and analyzed by light microscopy.

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primigenius, *Mus musculus*, *Drosophila melanogaster* and *Caenorhabditis elegans*. The identity of the invertebrate orthologs with the human GDP-fucose transporter ranges between 35 and 55% (Web Fig. B).

The analysis of protein topology for the human GDP-fucose transporter predicts 10 transmembrane domains with the N and carboxy termini exposed to the cytosol (Fig. 3). The four transmembrane domains 3, 4, 7 and 9 are highly conserved. It should be noted that the transmembrane domains 4 and 9 are highly hydrophilic and are thereby missed by standard algorithms for identification of membrane-spanning regions. Northern-blot analysis revealed several RNA species ranging from 1.3 kb to 6.4 kb, expressed in variable amounts and patterns in all tissues examined. Expression was highest in striated muscles and liver with a predominant 3.4-kb RNA species. Size and amounts of transcripts in fibroblasts and EBV-transformed lymphoblasts from patient A.C. and two other LAD II patients (M. and Y.W.) were comparable to those of controls (Web Fig. C).

For detection of mutations, we analysed the GDP-fucose transporter transcripts of the patients by sequencing overlapping RT-PCR-amplified fragments derived from fibroblast and lymphoblast RNA. In patient A.C., of Turkish origin, we identified a C→T transition at nt 439 within the coding region. The same mutation was detected in patient A.C., as described in an accompanying report¹⁰. This transition results in a missense change from arginine to cysteine at amino acid 147 (R147C), a residue conserved between all orthologs and residing in a highly conserved stretch of 16 amino acids (Fig. 4, top). Arginine 147 is part of the fourth transmembrane domain, which is distinguished by the presence of the arginine and several other hydrophilic residues. Genomic sequencing showed the homozygosity of the patient and heterozygosity of both parents for the R147C mutation. We did not detect the 439C→T transition in any of 40 chromosomes from controls. To distinguish between a polymorphism and a disease-causing mutation, we transiently expressed D5 cDNA carrying the 439C→T transition in fibroblasts from patient A.C. Expression of the 439C→T allele did not correct the fucosylation defect (Fig. 5b).

The two other people with LAD II, Y.W. (ref. 7) and M. (A.E., unpublished data), are from unrelated Arab families in Israel and present a more severe growth defect and mental retardation than patient A.C. Sequencing fragments amplified by RT-PCR revealed a 923C→G transversion in both Arab patients. Genomic sequencing showed homozygosity for the 923C→G transversion, which was not detectable in 30 chromosomes from controls. The 923C→G transversion leads to a missense change from threonine

to arginine at amino acid 308 (T308R). Threonine 308 is conserved among all GDP-fucose transporter orthologs, resides in a highly conserved stretch of 22 amino acids (Fig. 4, bottom) and is part of the ninth transmembrane domain. The latter shares with transmembrane domain 4 the presence of hydrophilic residues.

It is interesting to note that the two amino acids (R147 and T308) changed by mutations in LAD II patients are located in highly conserved transmembrane domains with an unusually high hydrophilicity. This points to a role of these residues in the gating of GDP-fucose. Supplementation of fucose led to a substantial clinical improvement and correction of the hypofucosylation in the patient homozygous for the R147C mutation¹¹, whereas it was of no benefit to the patients homozygous for the T308R mutation (A.E., unpublished data). The response to fucose supplementation indicates that the R147C mutation leads to a reduced affinity of the transport for GDP-fucose, which can be compensated for, in part, by elevating cellular GDP-fucose levels.

Note: supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

Methods

Cell lines and cell culture. The fibroblasts from patient A.C. (ref. 4), his parents and controls were maintained at 37 °C, under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% fetal calf serum (FCS, PAN Biotech GmbH). EBV-transformed lymphoblasts from the patients M. (A.E., unpublished data) and Y.W. (ref. 8) were maintained in RPMI medium (Gibco BRL) containing 10% FCS, which was heat-inactivated at 56 °C for 30 min. The ecotropic packaging cell line φNX-Eco (ATCC) and the amphotropic packaging cell line RetroPack PT67 (Clontech) were cultured in DMEM/10% heat-inactivated FCS at 37 °C under 5% CO₂ unless otherwise stated.

Human liver retroviral cDNA library and pLIB-EGFP vectors. The human liver retroviral cDNA library is commercially available (Clontech). Upon transfection into a packaging cell line, the Moloney murine leukemia virus (MoMuLV)-derived vector pLIB expresses a transcript containing the long terminal repeats, the ψ+ packaging signal and the gene of interest cloned into the MCS of the vector. The library consists of 8.0×10⁶ independent clones with an average cDNA size of 1.6 kb and a cDNA range of 0.6–5.0 kb.

Fig. 3 Predicted topology of the GDP-fucose transporter. Highlighted in light and dark gray are the highly conserved transmembrane domains 3, 4, 7 and 9. Transmembrane domains 4 and 9 (dark gray) have an unusual high hydrophilicity and were missed by standard algorithms.

Fig. 4 Amino acid changes in the GDP-fucose transporter of patient A.C. (top), and of patients M. and Y.W. (bottom). The amino acid residues exchanged by the missense mutations are highlighted by gray boxes; the non-conserved residues in the surrounding sequences by thin boxes.

The efficiency of transfection and transduction events was monitored with the reporter vector pLIB-EGFP (enhanced green fluorescent protein; Clontech). For the import assay, a 1.2-kb fragment of D5 representing the coding sequence (nt -44 to nt 1,123) was amplified by PCR using primers F6 and R5 (Web Methods). The resulting fragment was purified (Qiagen, PCR Purification kit) and cloned into pGEM-T Easy vector (Promega). After checking the accuracy of the fragment by sequencing, we subcloned the 1.2-kb product into the MoMuLV-derived vector pLNCX2 (Clontech).

Site-directed mutagenesis. We inserted the R147C mutation into pLNCX2-D5 using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with the primers MutR147C-F (5'-CTTCTACAATGTGGGCTGCTCACTCACCACCGTC-3') and MutR147C-R (5'-GACGGTGGTGAGTGAGCAGCCACATGTGTAAG-3').

Production of retroviruses. We seeded ϕ NX-Eco cells (0.75×10^6 cells) onto 60-mm dishes 1 day before the transfection. Transient transfection by FuGENE6 reagent was carried out according to the manufacturer's protocol (Roche). The cells were cultured for 24 h at 37 °C before shifting to 33 °C for a further 16 h. The ecotropic retroviral supernatant was filtered through a 0.45- μ m-pore filter (Sartorius), supplemented with Polybrene (6 μ g/ml; Sigma), then used to infect PT67 cells that had been seeded 24 h before infection at 0.75×10^6 cells/60-mm dish. After 24 h, the ecotropic supernatant was removed and the PT67 cells were harvested by trypsinization, transferred to 150-mm dishes and incubated at 37 °C for 12 h followed by an incubation at 33 °C for 16 h. The supernatant with the amphotropic retroviral particles was collected, filtered, supplemented with Polybrene (2 μ g/ml) and used to infect the patient fibroblasts, which had been seeded 48 h before infection at 3.0×10^5 cells/100-mm dish. After 16 h, we replaced the amphotropic retroviral supernatant by fresh DMEM/10% heat-inactivated FCS. After incubation for 8 h the infection procedure of the fibroblasts was repeated twice following the same protocol. After the final infection, the cells were grown for 72 h in DMEM/10% heat-inactivated FCS before staining with fucose-specific lectin or assaying for nucleotide sugars import. Control experiments using the pLIB-EGFP viral particles established that 30–50% of the fibroblasts expressed the retrovirally transduced green fluorescent reporter protein. We obtained stable cell lines by infecting fibroblasts of the patient with the pLNCX2 vector carrying the appropriate cDNA insert and subsequent selection with Geneticin (0.4 mg/ml; Gibco BRL) in DMEM/10% heat-inactivated FCS for 10 d.

Histochemical staining with AAL. Retrovirally transduced fibroblasts of the patient were washed three times with PBS (10 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4, 150 mM NaCl), fixed in 50% methanol/50% acetone for 2–3 min with gentle shaking and air-dried for 10 min. Fixed cells were washed twice with PBS and non-specific binding-sites were blocked by incubating the cells with 1% BSA (Sigma) in PBS for 45 min. The cells were then incubated for 30 min with 2 μ g/ml biotinylated AAL (Vector Laboratories) in PBS/1% BSA and then for 30 min with alkaline phosphatase polymer labeled streptavidin (Sigma) at 2 μ g/ml in PBS/1% BSA. Finally the cells were equilibrated (in 0.1 M Tris, pH 9.5, 0.1 M NaCl, 5 mM MgCl_2) and

Fig. 5 Phenotypic correction of patient fibroblasts. Fibroblasts from patient A.C. transiently expressing the D5 wild-type allele of the GDP-fucose transporter (a) or the 439C→T (b) or stably expressing the D5 wild-type allele (c) were histochemically labeled with biotinylated AAL and analysed by light microscopy.

incubated for 5–10 min at RT with Nitroblue tetrazolium (0.4 mM; Sigma) and 5-bromo-4-chloro-3-indolylphosphate-p-toluidinium (0.38 mM, Sigma) in the same buffer. All solutions were filtered through a 0.2- μ m-pore filter (Sartorius) before use and levamisole (2 mM, Sigma) was added to reduce background staining.

GDP-[^{14}C]fucose and UDP-[^3H]galactose import. The import of GDP-[^{14}C]fucose and UDP-[^3H]galactose into Golgi-enriched vesicles from retrovirally transduced fibroblasts was carried out as described³.

Mutation detection and genomic sequencing. Total RNAs from fibroblasts of the patient A.C. and a control cell line, and from EBV-transformed lymphoblasts of patient M., patient Y.W. and control cell lines were prepared according to the manufacturer's instructions (RNeasy Mini kit, Qiagen). Human brain and placenta RNA were purchased (Clontech). On these RNAs, we carried out nested RT-PCR reactions by using Omniscript reverse transcriptase (Qiagen) and HotStar Taq DNA polymerase (Qiagen) according to the manufacturer's instructions. We sequenced the purified RT-PCR products (PCR Purification kit, Qiagen) using BigDye Terminator cycle sequencing kit (ABI PRISM) according to the manufacturer's instructions and ran the sequencing reactions on a 310 Genetic Analyzer (ABI PRISM). (For information describing the primers and PCR conditions, see Web Methods.)

We prepared genomic DNA from fibroblasts and from peripheral blood leukocytes using standard procedures¹². PCR and sequence analysis of the mutations, wild-type alleles and 5' UTRs (Web Methods; see also Web Figs. A–G).

Northern-blot analysis. Total RNA was prepared from fibroblasts and EBV-transformed lymphoblasts of control and patients, separated and blotted onto nitrocellulose membranes. This and a human northern blot from 12 tissues, including brain, heart skeletal, muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and peripheral blood leukocytes (Clontech) were hybridized with a cDNA probe corresponding to nt 18–1,173 of clone D5 (pLIB-D5) and a β -actin cDNA probe as control for RNA loading. The probes were labeled with [$\alpha^{32}\text{P}$]dCTP using the

RediPrimeII kit (Amersham Pharmacia Biotech) The membranes were pre-incubated with salmon sperm DNA (0.2 µg/µl) and hybridized for 16 h at 42 °C in Rapid-hyb buffer (Amersham Life Science). The membranes were washed in 2×SSC, 0.1% SDS for 10 min at RT and in 0.2×SSC, 0.1% SDS for 15 min at 65 °C. For stripping, the membranes were boiled for 5 min in 0.1% (w/v) SDS and washed twice with 2×SSC before reprobing with a β-actin probe for control of RNA loading.

Web-based databases and sequence analysis. For homology searches we used BLAST and Psi-Blast from the server at NCBI (<http://www.ncbi.nlm.nih.gov/blast/>). The putative protein was analysed by Protean (DNA Star) to obtain a Kyte-Doolittle hydrophilicity plot. Multiple alignments were done with ClustalW (<http://www.ebi.ac.uk/clustalw/>). Predictions of the GDP-fucose transporter membrane topology were made mainly on the basis of calculations with the programs TMpred (ref. 13) and TMhmm (ref. 14).

GenBank accession numbers for GDP-fucose transporters. *Homo sapiens*: D5 cDNA, AF326199; D5 mRNA, NM_018389; ESTs, BE279067, AU139304. *B. primigenius* EST, BE683757; *S. scrofa* EST, BE232731; *M. musculus* EST, AI451820, AA244848, AW762672; *Drosophila*, AAF54215; *C. elegans*, CAA94748, CAB60293.

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