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Mutations in the *MGAT2* Gene Controlling Complex N-Glycan Synthesis Cause Carbohydrate-Deficient Glycoprotein Syndrome Type II, an Autosomal Recessive Disease with Defective Brain Development

Jenny Tan,[1] James Dunn,[2] Jaak Jaeken,[4] and Harry Schachter[1,3]

[1]Department of Biochemistry Research, The Hospital for Sick Children, [2]Visible Genetics Incorporated, and [3]Department of Biochemistry, University of Toronto, Toronto; and [4]Department of Biochemistry Research, University Hospital Gasthuisberg, and Department of Pediatrics, University of Leuven, Leuven, Belgium

Summary

Carbohydrate-deficient glycoprotein syndrome (CDGS) type II is a multisystemic congenital disease with severe involvement of the nervous system. Two unrelated CDGS type II patients are shown to have point mutations (one patient having Ser→Phe and the other having His→Arg) in the catalytic domain of the gene *MGAT2*, encoding UDP-GlcNAc:α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnT II), an enzyme essential for biosynthesis of complex Asn-linked glycans. Both mutations caused both decreased expression of enzyme protein in a baculovirus/insect cell system and inactivation of enzyme activity. Restriction-endonuclease analysis of DNA from 23 blood relatives of one of these patients showed that 13 donors were heterozygotes; the other relatives and 21 unrelated donors were normal homozygotes. All heterozygotes showed a significant reduction (33%–68%) in mononuclear-cell GnT II activity. The data indicate that CDGS type II is an autosomal recessive disease and that complex Asn-linked glycans are essential for normal neurological development.

Introduction

Carbohydrate-deficient glycoprotein syndromes (CDGS), first reported in 1980 (Jaeken et al. 1980), are genetic multisystemic diseases characterized by defective protein-bound carbohydrate and moderate to severe malformation of the nervous system (Blennow et al. 1991; Jaeken et al. 1991; Stibler et al. 1991; Hagberg et al. 1993; Jaeken and Carchon 1993; Jaeken et al. 1993*a*). Four variants have been described. At least 180 patients with CDGS type I have been reported, whereas CDGS types II (Ramaekers et al. 1991; Jaeken et al. 1993*b*, 1994; Charuk et al. 1995), III (Stibler et al. 1993) and IV (Stibler et al. 1995) are, to date, represented by only two families each. Several laboratories have provided evidence that CDGS type I may be due to defective mannose incorporation into dolichol pyrophosphate oligosaccharide, the precursor of Asn-linked glycans (N-glycans) (Wada et al. 1992; Yamashita et al. 1993*a*, 1993*b*; Knauer et al. 1994; Powell et al. 1994; Yasugi et al. 1994; Paneerselvam and Freeze 1995); it has now been shown that phosphomannomutase, which converts mannose-6-phosphate to mannose-1-phosphate, is markedly deficient in four patients with this disease (Van Schaftingen and Jaeken 1995). We have recently demonstrated a severe deficiency in the activity of UDP-GlcNAc:α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnT II; E.C.2.4.1.143) in fibroblast extracts from two CDGS type II patients (Jaeken et al. 1994) from unrelated Iranian (patient A) and Belgian (patient B) families and in mononuclear-cell extracts from patient B (Charuk et al. 1995).

GnT II is essential for the normal assembly of complex Asn-linked glycans (Schachter 1986). The gene for human GnT II (*MGAT2*) has been cloned and localized to chromosome 14q21 (Tan et al. 1995). Southern analysis indicated that there is only a single copy of *MGAT2*. The entire coding region was shown to be on a single exon. GnT II is a Golgi apparatus–localized type II membrane-bound protein with a domain structure typical of all glycosyltransferases cloned to date—namely, a short amino-terminal cytoplasmic domain, a combined signal/anchor transmembrane domain, a "stem" region, and a large lumenal carboxy-terminal catalytic domain (Paulson and Colley 1989; Schachter 1994). We now show that the lack of GnT II activity in the two CDGS type II patients is due to a point mutation in the catalytic domain of GnT II, Ser290Phe (TCC→TTC) in patient A and His262Arg (CAC→CGC) in patient B. We also describe simple restriction-endonuclease methods for de-

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Address for correspondence and reprints: Dr. Harry Schachter, Department of Biochemistry Research, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada. E-mail: harry@sickkids.on.ca

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Table 1

Synthetic Oligodeoxynucleotides Used as PCR Primers

Primer Name[a]	Sequence[b]
568F	5′-GGCCAGTTCCGACCGTGACA-3′
679F	5′-GACCATGAGGTTCCGCATCTACAAAC-3′
679BacF	5′-CGATAAGGATCCGACCATGAGGTTCCGCATCTACAAAC-3′
1234F	5′-CCCTAACGAGTTTCCAGGTAG-3′
1307BR	5′-biotin-CCAATTTCAAAGCGGCATTCTT-3′
1427F	5′-CGAGATTATGCTGGCCTTATACTTTTCCTAGAAGACGATC-3′
A1467GF	5′-CCTAGAAGAGGATCGCTACTTAGCCCC-3′
T1467CR	5′-GGGGCTAAGTAGCGATCCTCTTCTAG-3′
C1551TF	5′-GTGATGTTCTCTTCCTGGGGACCTATAGTG-3′
G1551AR	5′-ATAGGTCCCCAGGAAGAGAACATCACATTC-3′
1604F	5′-GATGTGAAAACTTGGAAATCC-3′
1633R	5′-GTGCTCTGTGGATTTCCAAGTTTTCACATC-3′
1635R	5′-TTGTGCTCTGTGGATTTCCAAGTTTTCACATC-3′
1729BR	5′-biotin-AGTCCAGTCCCAGTTATAATC-3′
2046R	5′-CGCTTTTGTAACTGTGATTTTCACTGC-3′
2046BacR	5′-CGAATTCCATGGCGCTTTTGTAACTGTGATTTTCACTGC-3′
2294R	5′-AAAATTTCCCCTCTTCTCTCA-3′
2294BR	5′-biotin-AAAATTTCCCCTCTTCTCTCA-3′

[a] Primer names reflect either the nucleotide position or the mutation. The nucleotide positions are numbered as previously published (Tan et al. 1995), with the ATG start codon at position 683 and with the TGA stop codon at position 2024. The nucleotide position is either the location on the genomic sequence of the first nucleotide at the 5′ end of the primer or the location of the mutation. F = forward primer; R = reverse primer; Bac = primer for cloning into baculovirus transfer vector; and B = 5′-biotin label.

[b] Underlined bases in 1427F, A1467GF, T1467CR, C1551TF, and G1551AR show the points of inserted mutations; and underlined bases in 679BacF and 2046BacR show the inserted *Bam*HI and *Nco*I restriction-enzyme cleavage sites, respectively.

tecting these two mutations. This is both the first CDGS to be characterized at the gene level and the first demonstration that complex N–glycan synthesis is essential for normal human brain development.

Material and Methods

Material

Oligodeoxynucleotides (table 1) were synthesized on a Pharmacia DNA synthesizer and were purified by the cartridge method (Hospital for Sick Children–Pharmacia Biotechnology Centre, Toronto). The following materials were purchased from the indicated sources: restriction endonucleases and T4 ligase (Pharmacia); Triton X-100 (Sigma); aprotinin, leupeptin, and aminoethylbenzene sulfonyl fluoride (ICN Biomedicals); linearized BaculoGold virus DNA and cationic liposomes (PharMingen); and *Spodoptera frugiperda* (Sf9) insect cells, nickel ProBond resin, and pBlueBacHis B (Invitrogen). *Escherichia coli* DH5aF (Gibco Laboratories or BRL) was used for all DNA amplifications. Both PCR using either pfu polymerase (Stratagene) or *Taq* polymerase (Cetus) and all other molecular biology procedures were performed by standard techniques using commercially available reagents and kits (Sambrook et al. 1989).

Fibroblasts and Mononuclear Cells

The two CDGS type II patients, a girl (patient A) and a boy (patient B), came from unrelated Iranian and Belgian families, respectively. Both patients had a similar facial dysmorphy, a ventricular septal defect, and severely retarded psychomotor development (Jaeken et al. 1993*b*, 1994). Isoelectrofocusing of serum transferrin showed that tetrasialotransferrin was nearly absent compared with that in controls and that there was a large increase of disialotransferrin carrying two truncated biantennary N-acetyllactosaminic glycans in each of which the trisaccharide element sialyl(α2-6)Gal(β1-4)GlcNAc(β1-2) attached to the (α1-6)-linked Man residue of the N-glycan core was missing (Jaeken et al. 1994). This is consistent with the finding that fibroblast extracts from the two CDGS type II patients A and B showed a reduction of >98% in GnT II (Jaeken et al. 1994) and that patient B had no detectable GnT II in his mononuclear-cell extract (Charuk et al. 1995).

Fibroblasts from patients A and B were maintained in culture and were harvested as described elsewhere (Jaeken et al. 1994). Blood was drawn in Belgium from CDGS type II patient B (donor 1; fig. 1), from 23 clinically normal blood relatives of patient B (donors 2, 3, 5–17, and 38–45; fig. 1), and from 21 normal controls

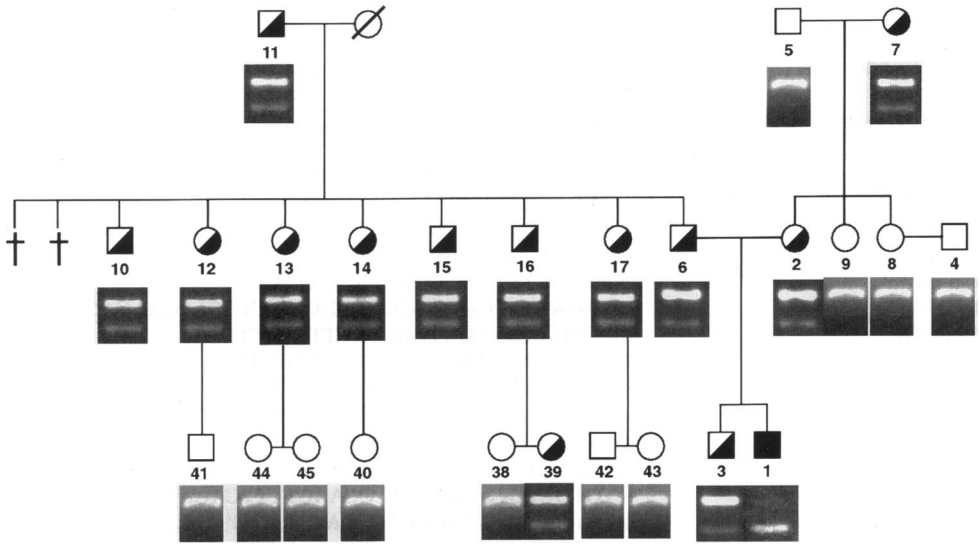

Figure 1 CDGS type II patient B's family tree and restriction-endonuclease analysis. The numbers are arbitrary assignments to the blood donors. Squares denote males; and circles denote females. The completely blackened square represents patient B; the half-blackened symbols represent heterozygotes, as determined by restriction-endonuclease analysis; and the unblackened symbols represent individuals in whom the CDGS type II genotype is absent. Both the symbol with a diagonal slash through it (∅) and the two crosses (†) denote that the individual is deceased. Underneath the symbols are shown the bands produced by electrophoresis of *Pvu*I digests of 207-bp segments of the GnT II gene that are produced by PCR as described in the text. The normal allele is not digested, whereas the mutant allele is digested into 168-bp and 39-bp bands. Only the 168-bp band is shown. Patient B shows complete digestion of the 207-bp PCR product, whereas heterozygotes show only partial digestion. Twenty-one unrelated blood donors were normal homozygotes, as determined by this test (not shown). Mononuclear-cell extracts from all 13 heterozygotes have GnT II levels that are 32%–67% lower than normal levels (average, 50.1% ± 10.7% SD) (Charuk et al. 1995; present study).

(donors 4 and 18–37; 13 males and 8 females) unrelated by blood to the patient. The blood was drawn into tubes containing acid-citrate-dextrose and was shipped on ice packs to Toronto, and mononuclear cells were prepared with Ficoll-Paque (Pharmacia) as described elsewhere (Charuk et al. 1995). The use of human materials was approved by review boards of the Hospital for Sick Children, Toronto, and of University Hospital Gasthuisberg, Leuven, Belgium, and informed consent was obtained from all donors.

Mutational Analysis by Direct Single-Strand DNA Sequencing

Since the entire coding region of human GnT II is on a single exon (Tan et al. 1995), PCR can be used to amplify the coding region by use of genomic DNA as a template. Genomic DNA (fibroblasts from two controls and from the two CDGS type II patients and mononuclear cells from patient B's brother and parents) was amplified into three overlapping fragments, of 740 bp, 496 bp and 691 bp, by the following PCR primer sets, respectively (table 1): 568F-1307BR, 1234F-1729BR, and 1604F-2294BR. Biotin-labeled PCR product was purified by using Streptavidin-coated Dynabeads M-280 (Dynal) as indicated by the manufacturer. The biotinylated and nonbiotinylated DNA strands were separated by NaOH treatment, and the nonbiotinylated strand was purified by ethanol precipitation. Both strands were used, without subcloning, for automated fluorescent sequencing on an ALF sequencer (Pharmacia) with the AutoRead T7 sequencing kit (Pharmacia) and synthetic 5′-fluorescein-oligodeoxynucleotide primers.

PCR-Mediated Site-Directed Mutagenesis

All PCR primers are shown in table 1. Two overlapping PCR fragments, of 1,068 bp and 1,061 bp (PCR1 and PCR2, respectively), were amplified from the coding region of the normal GnT II gene by plasmid pHG30 (Tan et al. 1995) as a template and primer sets 568F-1635R and 1234F-2294R, respectively. The PCR products were purified by electrophoresis.

C1551T mutation.—Primer sets C1551TF-2046R and 679F-G1551AR were used to amplify overlapping 508-bp and 886-bp products, by use of 2,000-fold-diluted PCR2 and PCR1, respectively, as templates. PCR products were purified, mixed, denatured at 95°C for 6 min, and reannealed at 45°C for 30 min. The annealed DNA was extended and amplified by PCR using primers 679BacF and 2046BacR with *Bam*HI and *Nco*I sites at their respective 5′-ends for directional subcloning into a baculovirus transfer vector (Tan et al. 1995).

A1467G mutation.—Primer sets A1467GF-2046R and 679F-T1467CR were used to amplify overlapping 594-bp and 801-bp products with 2,000-fold diluted

PCR2 and PCR1, respectively, as templates. PCR products were purified, mixed, denatured, reannealed, extended, and amplified as described above.

Expression of Mutant and Nonmutated GnT II Proteins

The two PCR products containing the respective point mutations were subcloned into the *Bam*HI-*Nco*I site of the baculovirus transfer vector pBlueBacHis B downstream from and in frame with the vector's ATG start site, and the sequence was confirmed (Sanger et al. 1977) by using a T7 sequencing kit (Pharmacia). Each plasmid construct (60 ng) was cotransfected with 14 ng of linearized BaculoGold virus DNA, by use of cationic liposomes (Baculogold Transfection kit; PharMingen), into 1×10^5 Sf9 cells (Tan et al. 1995). A control transfection was performed with pBlueBacHis B containing nonmutagenized GnT II insert. Recombinant baculovirus was collected from the culture medium after 6 d at 28°C and was used to infect 6×10^7 Sf9 cells at a multiplicity of infection of 2 plaque-forming units/cell. Cells were harvested after 3 d; were lysed in 0.1 ml PBS containing 2% Triton X-100, 0.2 M NaCl, and protease inhibitors (aprotinin, leupeptin, and aminoethylbenzene sulfonyl fluoride); and were assayed for GnT II activity (Tan et al. 1995). GnT II was partially purified on nickel ProBond resin as described elsewhere (Tan et al. 1995). Lysates of uninfected Sf9 cells (6×10^7) were similarly treated, as negative controls. Eluates from the nonmutated GnT II preparation were assayed for GnT II activity, and enzyme-containing fractions were pooled and concentrated 100-fold by microcon-30 (Amicon). No GnT II activity was detected for either of the mutant preparations, and nickel ProBond resin eluates from these preparations and from the negative controls therefore were pooled and concentrated by the same method as was used for the active preparation.

Western Blot Analysis

Recombinant GnT II proteins and negative controls, after partial purification on Nickel ProBond resin, were subjected to 12.5% SDS-PAGE (Laemmli 1970) and were transferred to an Immobilon-P PVDF membrane (0.45 microns; Millipore). Western blot analysis was performed with 1:5,000-diluted Anti-Xpress antibody (directed against the enterokinase cleavage sequence of the recombinant proteins) as described by the manufacturer (Invitrogen). The procedure was repeated with crude Sf9 cell lysates containing recombinant GnT II proteins. Proteins were visualized by treatment with 1:2,500-diluted alkaline phosphatase–conjugated anti-mouse IgG (Sigma), followed by staining with bromochloroindolyl phosphate and nitro blue tetrazolium (Promega). The stained membranes were scanned (UVP scanner), and signal intensities were semiquantitated in square pixels by the computer program provided by the scanner manufacturer (UVP-Grab IT).

Mutation Screening by Restriction-Endonuclease Analysis

Genomic DNA from mononuclear cells was amplified by primers 1427F and 1633R (table 1), and the 207-bp product was purified and used, without prior subcloning, for restriction-enzyme analysis by electrophoresis on a 3% MetaPhor agarose gel (FMC Bioproducts), followed by ethidium bromide staining. The C1551T mutation of patient A created a restriction site CTCTTCN1/4 for *Ksp*632I (Boehringer Mannheim). When the 207-bp PCR product was treated with *Ksp*632I and analyzed by agarose gel electrophoresis, the normal allele was not digested, whereas the mutant allele gave two products, one of 126 bp and the other of 81 bp. The A1467G mutation of patient B does not create or destroy a known restriction-enzyme site. Since forward primer 1427F contained a G1462C mutation (underlined in table 1), a *Pvu*I restriction site (CGATCG) was created when the A1467G mutation was present. Digestion of the PCR product by *Pvu*I gave a 207-bp product for the normal allele and gave two bands, at 168 bp and 39 bp, for the mutant allele.

Northern Blot Analysis

Total RNA was extracted, by TRIzol reagent (Gibco Laboratories), from the fibroblasts of two controls and the two CDGS type II patients and was subjected to Northern analysis as described elsewhere (Tan et al. 1995), by a 510-bp [^{32}P]-labeled human GnT II genomic DNA probe generated by digestion of pHG36 with *Kpn*I and *Sac*I. RNA standards were detected by staining with methylene blue (Sambrook et al. 1989).

Results

Point Mutations in GnT II from CDGS Type II Patients

Direct sequencing in both directions of the entire GnT II coding region from two unrelated CDGS type II patients (fig. 2) identified two point mutations in the catalytic domain of GnT II, C1551T (Ser290Phe) in patient A and A1467G (His262Arg) in patient B. Both patients are homozygous for their respective mutations and therefore have inherited the same allele from both parents. The father of patient B shows one normal allele and one allele with the same mutation as is seen in patient B (fig. 2). The mother and brother of patient B show the same heterozygote nucleotide-sequence pattern as is seen in the father (data not shown). The data suggest that both pairs of parents may be consanguineous.

Site-Directed Mutagenesis of GnT II

To determine the effect of the identified mutations on the activity of human GnT II, the C1551T and A1467G

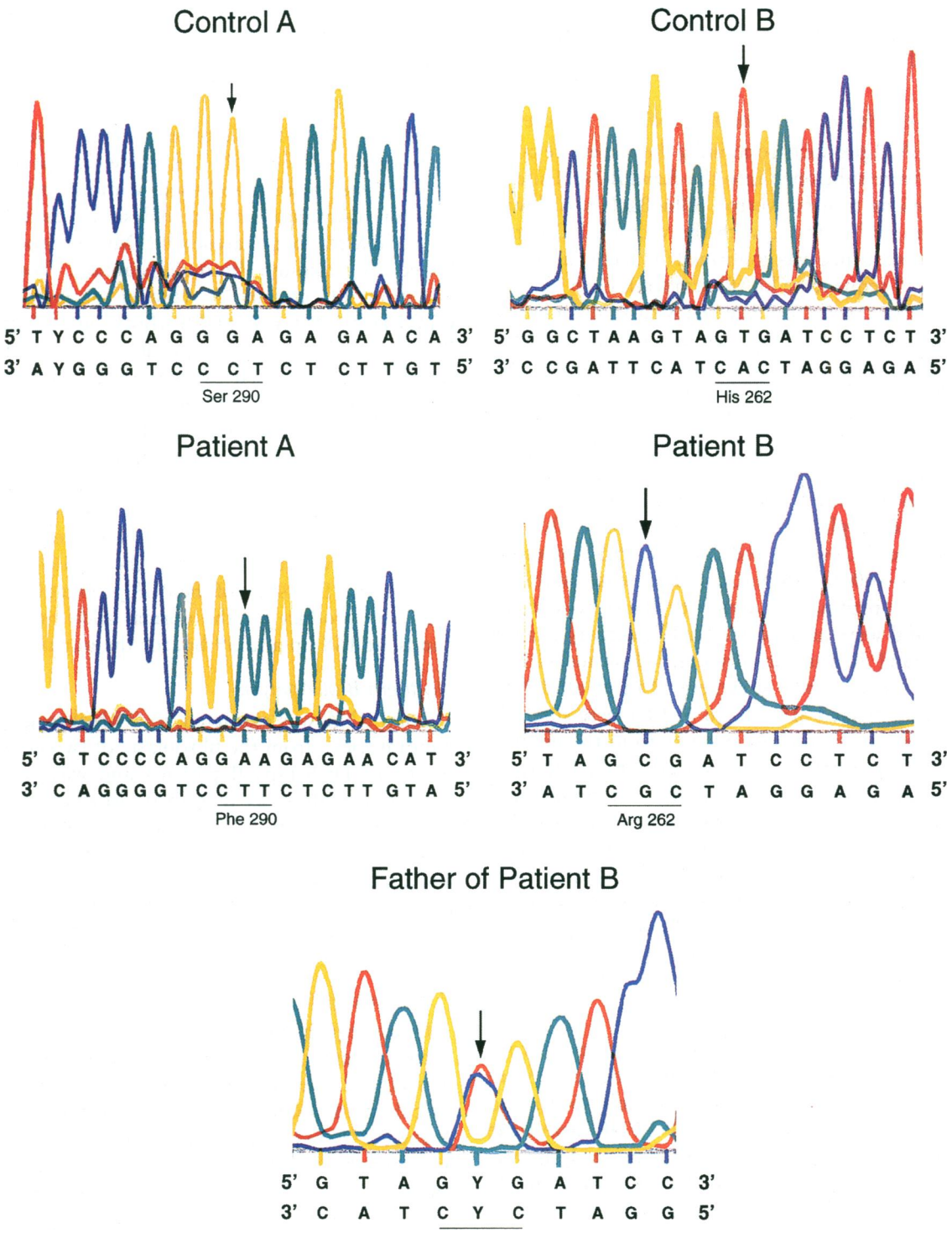

Figure 2 Nucleotide sequence analysis of the GnT II gene from a normal control, CDGS type II patients A and B, and patient B's father. The sequences are shown in the antisense direction. Control A shows the sequence around nucleotide position 1551, and control B shows that around position 1467. The only differences (*arrows*) from the control GnT II sequence are C1551T (G1551A; Ser290Phe) for patient A and A1467G (T1467C; His262Arg) for patient B. Patient B's father showed both a T and a C at position 1467.

substitutions were introduced into the normal *MGAT2* gene, followed by expression in the baculovirus/Sf9 system. The nonmutagenized gene was expressed under identical conditions as a positive control and produced an active enzyme (6.6 nmol/10 μl lysate/h) as described elsewhere (Tan et al. 1995). However, no enzyme activity was detected in cells transfected by either of the two mutant genes; the assay can detect ≥0.06 nmol/10 μl lysate/h (<1% of the control value). To determine whether the absence of GnT II activity in the mutants was caused by nonexpression of the protein, an antibody against the enterokinase cleavage sequence of the recombinant fusion proteins was used in Western blot analysis to detect and quantitate the amount of normal and mutant GnT II protein expressed (fig. 3). Both mutant proteins were expressed at ~8% of the level of normal GnT II expression, indicating that the mutations either interfere with transcription-translation or lower the stability of the protein in the baculovirus/Sf9 system; transcription is normal in human cells (see below), but whether there is a defect of either translation or protein stability in human cells remains to be established. Although the levels of expression in the baculovirus/Sf9 system were low, sufficient enzyme protein was produced to be readily detectable by enzyme-activity assays; since no enzyme activity was detected, the data show that the mutations not only have reduced protein expression but also have inactivated GnT II.

Restriction-Endonuclease Analysis of MGAT2

Restriction-endonuclease methods for detecting the two mutations were developed. PCR using a mismatched primer was used to introduce a *Pvu*I site into a 207-bp segment of the gene whenever the A1467G mutation was present. Mononuclear-cell DNA from patient B, 23 blood related family members, and 21 unrelated Belgian blood donors was tested by this method; and the results of this analysis are summarized in figure 1. Patient B is homozygous for the mutation whereas his asymptomatic brother, both parents, and 10 other relatives are heterozygotes; the other relatives and the unrelated donors were normal homozygotes. The C1551T mutation introduces a new restriction-endonuclease site for *Ksp*632I. Incubation, with *Ksp*632I, of the 207-bp PCR product from patient A resulted in 126-bp and 81-bp bands, whereas DNA from patient B and a normal control was not digested (not shown).

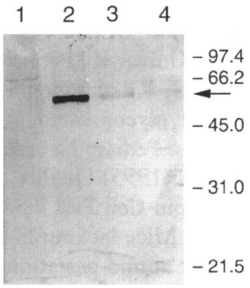

Figure 3 Western blot analysis of recombinant normal and mutant GnT II proteins after expression in the baculovirus/Sf9 system. The recombinant proteins were partially purified on a nickel resin and were detected by an antibody to the enterokinase cleavage sequence. Lane 1, Uninfected Sf9 cells. Lane 2, Nonmutated GnT II. Lane 3, S290F mutant GnT II. Lane 4, H262R mutant GnT II. The molecular masses of the three fusion proteins were ~56 kD (theoretical 55.3 kD [Tan et al. 1995]). Scanning of the blot indicated that the mutant proteins were expressed at 8% of the level of the normal protein. Molecular masses (in kD) are indicated. A similar Western blot was obtained with crude Sf9 lysates, indicating that poor expression of the mutant proteins was not due to losses during purification.

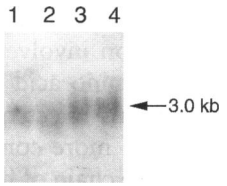

Figure 4 Northern blot analysis of total RNA isolated from fibroblasts from two controls (lanes 1 and 2) and CDGS type II patients A and B (lanes 3 and 4, respectively), by use of a human GnT II probe, as described in the text. All samples show a major band at ~3 kb.

Northern Analysis of CDGS Type II Fibroblasts

Northern analysis was performed on total RNA from cultured fibroblasts from two controls and the two CDGS type II patients A and B by use of a human GnT II probe (fig. 4). All four samples showed a 3-kb band at approximately the same intensity, indicating that neither mutation caused a defect in transcription.

Discussion

CDGS type II is a newly discovered genetic disease with severe neurological defects (Ramaekers et al. 1991; Jaeken et al. 1993*b*, 1994; Charuk et al. 1995). Only two families (represented by patients A and B, respectively) with this disease have been identified to date. We have shown elsewhere that fibroblasts from patients A and B have >98% reduction in UDP-GlcNAc:α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnT II) activity (Jaeken et al. 1994) and that patient B's mononuclear cells have no detectable GnT II activity (Charuk et al. 1995).

In the present work, we show that CDGS type II patients A and B are homozygous for two different *MGAT2* point mutations (S290F and H262R, respectively). Both mutations occur in the C-terminal catalytic domain (Schachter 1994, 1995) at locations that are

conserved between rat and human GnT II (Tan et al. 1995). The S290F mutation involves a change from a polar to a hydrophobic amino acid and might be predicted to have a deleterious effect on GnT II activity. The H262R mutation is a more conservative substitution, but the imidazole side chain of histidine is involved in the catalytic mechanisms of many enzymes, and the metal-binding property of histidine could be important for GnT II activity. Both mutant enzymes are poorly expressed (8% of normal) in the baculovirus/Sf9 system. Whether this is also true for humans remains to be established. Since Northern analysis of human CDGS type II fibroblasts showed no apparent abnormality, defective expression in humans, if it occurs, must be due either to defects in translation or to improper folding of the protein, leading to its destruction by the quality-control system of the cell (Hammond and Helenius 1995).

Although protein expression in the baculovirus/Sf9 system was relatively low, the amount of protein obtained would have been readily detected by our standard GnT II enzyme assay. Since no enzyme activity was detected with either of the mutant proteins, it can be concluded that both mutations inactivate GnT II. The phenotype of the CDGS type II patients therefore can be attributed to inactivation of GnT II by these mutations, although defective translation or proteolysis may also play an important role. It is of interest that, although the mutations in the two patients are different, they cause an identical phenotype. Analysis of patient B's family proves that CDGS type II is a recessive autosomal disease located at chromosome 14q21 and that the H262R mutation is not due to polymorphism. The inactivation of GnT II by the S290F mutation in patient A suggests that this mutation is also not due to polymorphism.

UDP-GlcNAc:α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I; E.C.2.4.1.101) is essential for the conversion of oligomannose-type Asn-linked glycans to hybrid and complex N-glycans (Schachter 1986). "Null" mice embryos lacking an active GnT I (Ioffe and Stanley 1994; Metzler et al. 1994) do not survive past 10.5 d of embryonic life and show many developmental defects particularly in the CNS. GnT II is essential for the biosynthesis of complex Asn-linked glycans (Schachter 1986, 1995), and CDGS type II patients with inactivating mutations of the GnT II gene develop severe multisystemic developmental abnormalities, especially in the nervous system, providing the first direct evidence that complex N-glycans are essential for normal human development. Studies with mice and studies with humans therefore indicate the importance of Asn-linked glycans in normal morphogenesis. Although there is a great deal of evidence suggesting that mediation of cell-cell interactions is the basis for the role of cell-surface glycoconjugates in embryogenesis and development (Fenderson et al. 1984, 1990; Eggens et al. 1989; Muramatsu 1992; Feizi 1993; Fukuda 1994), the detailed mechanisms of these processes remain to be determined.

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