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Mutations in the O-Mannosyltransferase Gene *POMT1* Give Rise to the Severe Neuronal Migration Disorder Walker-Warburg Syndrome

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Walker-Warburg syndrome (WWS) is an autosomal recessive developmental disorder characterized by congenital muscular dystrophy and complex brain and eye abnormalities. A similar combination of symptoms is presented by two other human diseases, muscle-eye-brain disease (MEB) and Fukuyama congenital muscular dystrophy (FCMD). Although the genes underlying FCMD (*Fukutin*) and MEB (*POMGnT1*) have been cloned, loci for WWS have remained elusive. The protein products of *POMGnT1* and *Fukutin* have both been implicated in protein glycosylation. To unravel the genetic basis of WWS, we first performed a genomewide linkage analysis in 10 consanguineous families with WWS. The results indicated the existence of at least three WWS loci. Subsequently, we adopted a candidate-gene approach in combination with homozygosity mapping in 15 consanguineous families with WWS. Candidate genes were selected on the basis of the role of the FCMD and MEB genes. Since *POMGnT1* encodes an O-mannoside N-acetylglucosaminyltransferase, we analyzed the possible implication of O-mannosyl glycan synthesis in WWS. Analysis of the locus for O-mannosyltransferase 1 (*POMT1*) revealed homozygosity in 5 of 15 families. Sequencing of the *POMT1* gene revealed mutations in 6 of the 30 unrelated patients with WWS. Of the five mutations identified, two are nonsense mutations, two are frameshift mutations, and one is a missense mutation. Immunohistochemical analysis of muscle from patients with *POMT1* mutations corroborated the O-mannosylation defect, as judged by the absence of glycosylation of α-dystroglycan. The implication of O-mannosylation in MEB and WWS suggests new lines of study in understanding the molecular basis of neuronal migration.

Introduction

Neuronal migration is a complex process by which the postmitotic neurons generated in the ventricular zone disperse and are positioned into six distinct layers in the mammalian neocortex. This process takes place during the 3rd and 4th mo of gestation and is characterized by an inside-out migration pattern, by which the successively newer neurons bypass the older ones to occupy a more exterior layer in the brain cortex (Marin-Padilla 1978). More than 25 neuronal migration disorders, resulting in death or improper positioning of the cortical neurons, have been described in humans (Lammens 2000). The deficient neuronal migration is accompanied by the absence of gyration of the brain surface, referred to as "smooth brain" or "lissencephaly."

Walker-Warburg syndrome (WWS [MIM 236670]) is a recessive disorder characterized by severe brain malformations, muscular dystrophy, and structural eye abnormalities. The brain manifests cobblestone lissencephaly with agenesis of the corpus callosum, cerebellar hypoplasia, hydrocephaly, and sometimes encephalocele (fig. 1). Cobblestone lissencephaly, also known as "cobblestone complex," is caused by neural overmigration, during neocortex lamination, that gives rise to disorganized cerebral and cerebellar cortexes and multiple coarse gyri with agyric regions. Two other human syndromes present with a similar association of symptoms: Fukuyama congenital muscular dystrophy (FCMD [MIM 253800]) and muscle-eye-brain dis-

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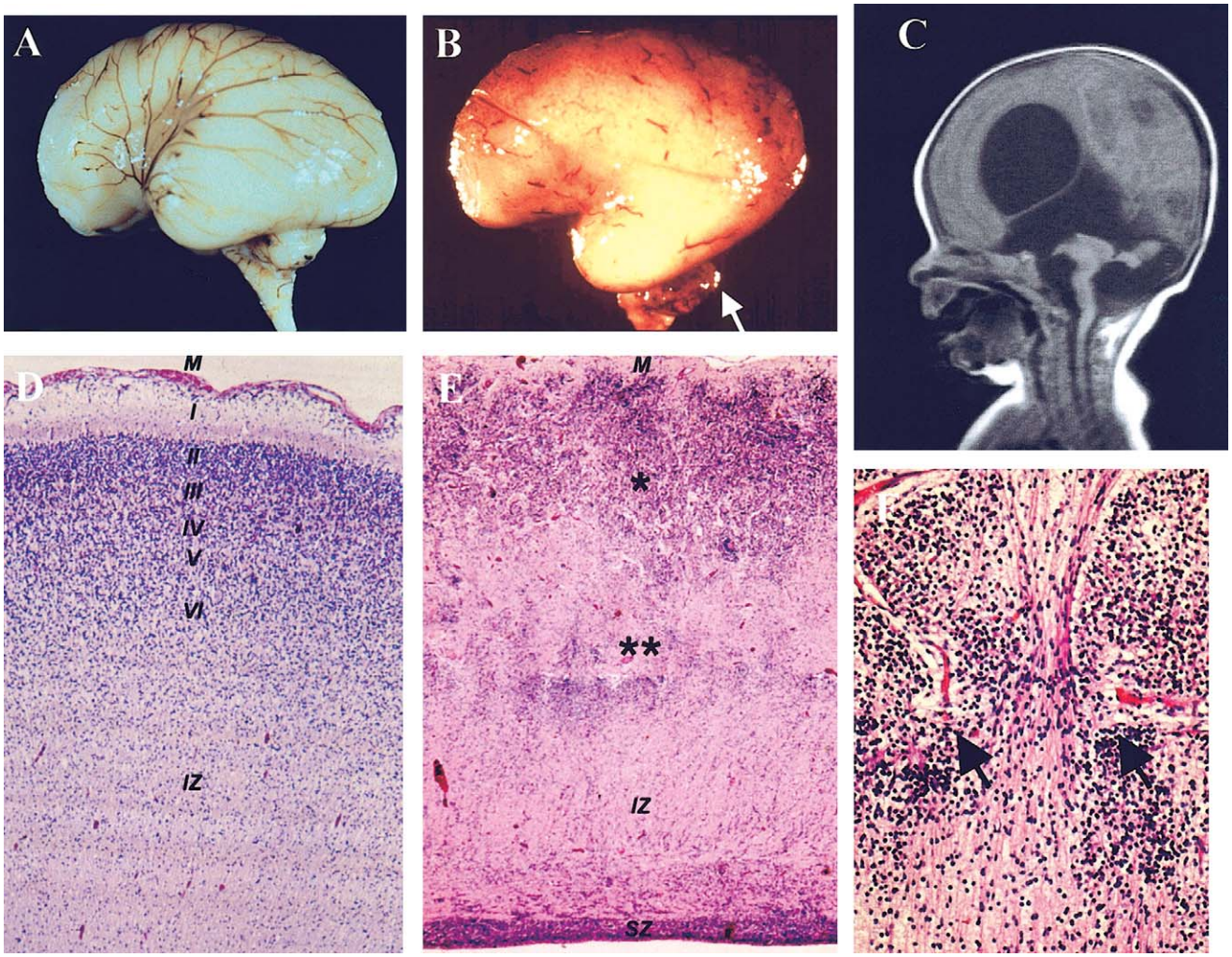


Figure 1 Brain abnormalities in patients with WWS who have POMT1 mutations. *A,* Normal brain of fetus at 21 wk gestation. *B,* Brain from an affected fetus from family 4 at 19 wk gestation. Note the rough cobblestone brain surface, the cerebellar hypoplasia (*arrow*), and the abnormal vasculature. *C,* Midsagital T1–weighted magnetic-resonance image from an affected child from family 2, showing enlarged ventricles, lack of corpus callosum, absence of pons, and rudimentary cerebellar vermis. *D–F,* Haematoxylin and eosin staining of paraffin-embedded brain sections. *D,* Fetal brain at 21 wk gestation, showing normal lamination of the cortex: meninges (*M*), lamina molecularis (*I*), lamina granularis externa (*II*), lamina pyramidalis (*III*), lamina granularis interna (*IV*), lamina ganglionaris (*V*), lamina multiformis (*VI*), and intermediate zone (*IZ*). *E,* Brain section from affected fetus from family 4, showing abnormal cortical lamination with disruption of the glia limitans (**), and protrusion of the migrating neuroblasts into the subarachnoid space (*). *SZ* = subventricular zone. *F,* Higher magnification of panel E, showing the subarachnoid-space invasion by glio-vascular neuronal tissue. Glia limitans is shown with an arrow.

ease (MEB [MIM 253280]). Clinical and genetic analysis has revealed that WWS represents an independent entity (Dobyns et al. 1989; Cormand et al. 2001). WWS is the most severe syndrome of the group, especially with regard to the brain phenotype. Life expectancy of patients with WWS is usually <1 year. In addition, recent linkage studies showed that WWS is not allelic to the other two syndromes (Cormand et al. 2001), but the loci for WWS have remained elusive.

Protein glycosylation is a highly complex mechanism by which sugars are sequentially added to proteins at the endoplasmic reticulum and the Golgi apparatus. This posttranslational process modulates protein stability, conformation, and function and has been implicated in diverse molecular recognition events, such as cell adhesion, growth, and differentiation (Kobata 1992; Varki 1993). The protein-attached glycans are divided into two groups on the basis of their linkage site: the N-glycans are linked to an asparagine residue of the target protein, whereas the O-glycans are attached through a serine or a threonine. Several O-linked glycans can be distinguished by the sugar moiety that O-links with the protein—for example, mannose, N-acetylgalactosamine, or fucose (Endo 1999). O-

mannosyl glycan synthesis is well studied in yeast, but little is known about this process in higher eukaryotes (Strahl-Bolsinger et al. 1999). Among the few O-mannosylated proteins that have been identified in mammals are α-dystroglycan, N-CAM, and tenascin-J1 (Wing et al. 1992; Chiba et al. 1997; Sasaki et al. 1998). All of the O-mannosyl glycans sequences identified, although diverse, share the common motif galactose-β-1, 4-N-acetylglucosamine-β-1, 2-mannose-O-Ser/Thr (Galβ1 4GlcNAcβ1 2Man-O-Ser/Thr). So far, O-mannose–linked glycosylation has been observed only in brain, peripheral-nerve, and muscle glycoproteins (Wing et al. 1992; Chiba et al. 1997; Yuen et al. 1997; Sasaki et al. 1998; Smalheiser et al. 1998; Chai et al. 1999). It has been estimated that 30% of all O-linked sugar chains in brain are O-mannose based (Chai et al. 1999).

Recently, the causative genes for FCMD (*Fukutin*) (Kobayashi et al. 1998) and MEB (*POMGnT1*) (Yoshida et al. 2001) have been cloned. The MEB gene product, POMGnT1, adds an N-acetylglucosamine residue to a preexisting protein O-linked mannose (Yoshida et al. 2001). The function of Fukutin is unknown, but it has been hypothesized to be a glycosyltransferase, based on amino acid homology with several phosphoryl-ligand transferases (Aravind and Koonin 1999). In addition, the highly homologous Fukutin-related protein (FKRP) is also a putative glycosyltransferase (Brockington et al. 2001*a*). Mutations in FKRP give rise to congenital muscular dystrophy with or without neural involvement (Brockington et al. 2001*a,* 2001*b*). Hypoglycosylation of α-dystroglycan has been observed in muscle tissue from patients with mutations in *POMGnT1, FCMD,* and *FKRP.* These observations indicate the implication of a common and crucial glycan synthesis process in these disorders.

In the present article, we describe that, in a proportion of patients with WWS, the disease is caused by mutations in the *POMT1* gene. This gene encodes O-mannosyltransferase 1, the enzyme that putatively catalyzes the first step in O-mannosyl glycan synthesis. These results demonstrate a key function of O-mannose–linked glycosylation in neural migration and open new lines of study in understanding the molecular basis of brain and muscle development.

Subjects, Material, and Methods

Subjects

All index patients ($n = 30$) were diagnosed through the documentation of cobblestone lissencephaly observed on brain imaging or postmortem investigation, as described elsewhere (Cormand et al. 2001). In addition, at least one of the following was present: eye malformations, congenital muscular dystrophy or elevated creatine kinase (CK), or occipital encephalocele. Affected siblings (including aborted fetuses) were diagnosed to be also affected if the diagnosis in the index case was secure and if at least one of the typical features was documented. The phenotypes of patients that had POMT1 mutations are described in more detail below.

The parents of family 1 are first cousins of Turkish origin and have been described elsewhere (family 6 in Cormand et al. 2001). After three spontaneous abortions, a male sibling was born presenting with severe hydrocephalus with dilatation of the third and fourth ventricle and minimal cortical development, no gyri visible, bifid cerebellum, and hypoplasia of the vermis and of the cerebellar hemispheres. A cerebellar cyst was observed. The corpus callosum appeared to be present. Microphthalmia of left eye and exophthalmia of right eye were noted. Hypoplastic genitalia were also present. Serum CK levels were highly elevated at more than 2,000 U/liter. The patient died at age 7 mo, and no DNA is available. A second child (an affected female sibling) died 15 min after birth. She presented with severe hydrocephaly, encephalocele, and bilateral cleft lip. DNA from this patient was used for genetic analysis.

Family 2 is a consanguineous family (first cousins) of Turkish origin living in Germany and has been described elsewhere (family 14 in Cormand et al. 2001). There is one healthy child, and there are three siblings diagnosed with WWS, a girl who died at age 3 years and two fetuses. The deceased girl had cobblestone lissencephaly, microphthalmia, buphthalmos, megalocornea, glaucoma, and retinal dysplasia. Serum CK was 1,700 U/liter. One fetus had an encephalocele. The brain of the other fetus is shown in figure 1.

Family 3 is also a consanguineous Turkish couple (first cousins) with one affected girl and two unaffected boys and has been described elsewhere (family 8 in Cormand et al. 2001). The girl died at age 2 mo. She presented with severe hydrocephalic dilatation of the lateral and third ventricles, hypoplasia of vermis and cerebellum, cyst formation in the posterior fossa, and a Dandy-Walker–like deformation of the fourth ventricles and brainstem. Eye malformations included bilateral buphthalmos, bilateral glaucoma, and hypertelorism. Serum CK levels were higher than 2,000 U/liter.

Family 4 is a Gypsy family from Italy and has been described elsewhere (Villanova et al. 1998). The patient is a boy with hydrocephalus and cobblestone lissencephaly with agyria and agenesis of the corpus callosum. Hypoplasia of the cerebellar vermis was seen in the posterior cranial fossa. Ophthalmological examination showed buphthalmos, retinal dysplasia, and lens opacities. Serum CK levels were >3,500 U/liter.

Patient 6 is an affected girl from unrelated Dutch par-

ents. She has hydrocephalus, cobblestone lissencephaly, cerebellar atrophy, bilateral microphthalmia, and frontal bossing. A previous pregnancy was terminated because of exencephaly.

The patients from family 7 are MZ twin girls from an unaffected, nonconsanguineous Austrian couple. Both present with encephalocele, pachygyria/agyria on magnetic-resonance imaging, large ventricles, cerebellar vermis hypoplasia/aplasia, corpus callosum hypoplasia, markedly raised CK, and cataract. One child has bilateral microtia.

Linkage Analysis

Genomic DNA was extracted, by standard methods, from peripheral-blood lymphocytes. In a first attempt to clone the WWS gene, we performed a 10-cM-average genomewide screening on 10 consanguineous families with WWS. For the POMT1 linkage analysis, we tested three microsatellite markers by PCR: D9S260, D9S64, and D9S1793. In brief, amplification was performed in a total volume of 20 μl (containing 40 ng of genomic DNA, 5 pmol of each primer, 1 U *Taq* DNA polymerase [Invitrogen], 250 μM of each dNTP, 1.5 mM $MgCl_2$ [or 1.25 mM, in the case of D9S64], 50 mM KCl, and 20 mM Tris-HCl [pH 8.4; pH 9.5 in the case of D9S64]). PCR conditions were as follows: 1 cycle at 94°C for 4 min; 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 4 min. Samples were resolved on 8% polyacrylamide sequencing gels and were developed by silver staining (Budowle et al. 1991).

Mutation Analysis

The 19 coding exons of the *POMT1* gene were amplified using specific primers for the 5′- and 3′-flanking intron sequences. The PCR conditions were optimized using the PCR Optimizer Kit (Invitrogen). The primer sequences and conditions are available on request. The amplicons generated were purified from agarose gels and were directly sequenced with the BigDye terminator kit (Perkin Elmer Applied Biosystems). Sequences were analyzed on an ABI3700 capillary sequencer (Perkin Elmer Applied Biosystems). Testing of the mutations and SNPs in the normal population was performed by SSCP analysis or by restriction-enzyme analysis in the event of the Gln303Stop (*Bfa*I) and Gln385Stop (*Pvu*II) mutations.

Immunohistochemistry

Skeletal-muscle biopsy specimens were shock-frozen in isopentane cooled in liquid nitrogen. Immunostaining of 6–8 μm cryosections was performed as described elsewhere (Kano et al. 2002) by using the following primary antibodies: affinity-purified polyclonal sheep antibodies directed against a 20-amino-acid C-terminal sequence of chick α-dystroglycan (Herrmann et al. 2000), monoclonal antibody (mAb) raised against α-dystroglycan (VIA4-1; Upstate Biotechnology), mAb anti-β-dystroglycan (clone 8D5; Novocastra), mAb anti–laminina-α2 (MAB 1922; Chemicon), and mAb anti–laminin-α2 directed against the 300-kDa subunit (4H8-2; Alexis). Sections were preincubated with PBS containing 5% BSA at pH 7.4, then were incubated with the primary antibodies overnight at 4°C, and subsequently were incubated with the secondary antibodies for 1 h at room temperature. Control sections with omission of the first antibody were run in parallel. Sections were examined and photographed on a Zeiss Axioplan fluorescence microscope.

Results

Genomewide Linkage Analysis

To unravel the genetic basis of WWS, we performed homozygosity mapping in 10 consanguineous families with WWS, using 10-cM-spaced genetic markers. In a first approach, we based our search on a one-locus premise. Some good candidate regions of common homozygosity were found, but further analysis denied the existence of a unique locus compatible with all families with WWS. Moreover, further studies based on a two-loci premise were negative and suggested the existence of at least three WWS loci. The high number of candidate loci and the minute amounts of patient samples available prompted us to combine the homozygosity mapping with a candidate gene approach. We focused on genes involved in glycosylation and investigated whether O-mannosyl glycan synthesis might underlie WWS. The first step of the synthesis of these glycans is executed by O-mannosyltransferases. As inferred from sequence homology with the O-mannosyltransferases Pmt1–Pmt7 from yeast (Strahl-Bolsinger et al. 1999), four putative human counterparts have been described: POMT1, POMT2, SDF2, and SDF2L1 (Hamada et al. 1996; Jurado et al. 1999; Fukuda et al. 2001). The protein domains of POMT1 and POMT2 show a similar arrangement to the yeast Pmts, whereas SDF2 and SDF2L1 lack the hypothetical catalytic domain located in the first 300 amino acids. We selected markers flanking the *POMT1* (D9S260 and D9S1793; on 9q34) and *POMT2* (D14S254 and D14S287; on 14q24) genes for homozygosity mapping in 15 consanguineous families. The results were especially promising in the case of *POMT1*. To substantiate the possible linkage at the *POMT1* locus, we tested an additional genetic marker, D9S64, which is located in intron 2 of the *POMT1* gene. Homozygosity was observed in patients from 5 of the 15 consanguineous families at this locus; only 1 of these 5 families was already included in the genomewide linkage analysis.

Human *POMT1* encodes a putative O-mannosyl-

Table 1

Mutations and Polymorphisms of *POMT1* in Families with WWS

			Haplotypes Associated with *POMT1* Mutations[b]							
Family (Origin)[a]	Consanguinity	Mutation	D9S260	c.1-6	D9S64	AA251	c.942	c.1148+16	c.2244+41	D9S1793
1 (Turkey)	Yes	c.226G→A (G76R)	186	T	105	R	C	G	C	182
2 (Turkey)	Yes	c.907C→T (Q303X)	182	T	107	R	C	G	C	182
3 (Turkey)	Yes	c.907C→T (Q303X)	182	T	107	R	C	G	C	182/172
4 (Italy)	No	c.2110InsG (V703fs)	184	G	111	Q	T	G	T	188
5a (Turkey)	Yes	NM	186	T	105	W	C	A	C	188
5b (Turkey)	Yes	NM	182	T	105	W	C	A	C	172
6a (Netherlands)	No	c.1283T→A (V428D)	182	T	105	R	C	G	C	182/172
6b (Netherlands)	No	c.2167InsG (G722fs)	182	T	103	R	C	G	C	182/172
7 (Austria)	No	c.1153C→T (Q385X)	182	T	105	R	C	G	C	186/178

Note.—NM = no mutation found.

[a] The chromosomes are identified by the pedigree code number, followed by "a" or "b" (which refer to each of the two haplotypes identified in a heterozygous patient; no letter is added if the patient is homozygous).

[b] Allelic associations of eight genetic polymorphisms (ordered from 5′ to 3′) for each of the families with linkage to *POMT1* are shown. D9S260, D9S64, and D9S1793 are dinucleotide repeats, and the others are novel SNPs. D9S64 is located in *POMT1* intron2, D9S260 is 3.1 cM toward the centromere, and D9S1793 is 3.9 cM toward the telomere. Note the shared haplotype for families 2 and 3, which carry the same nonsense mutation, Gln303Stop.

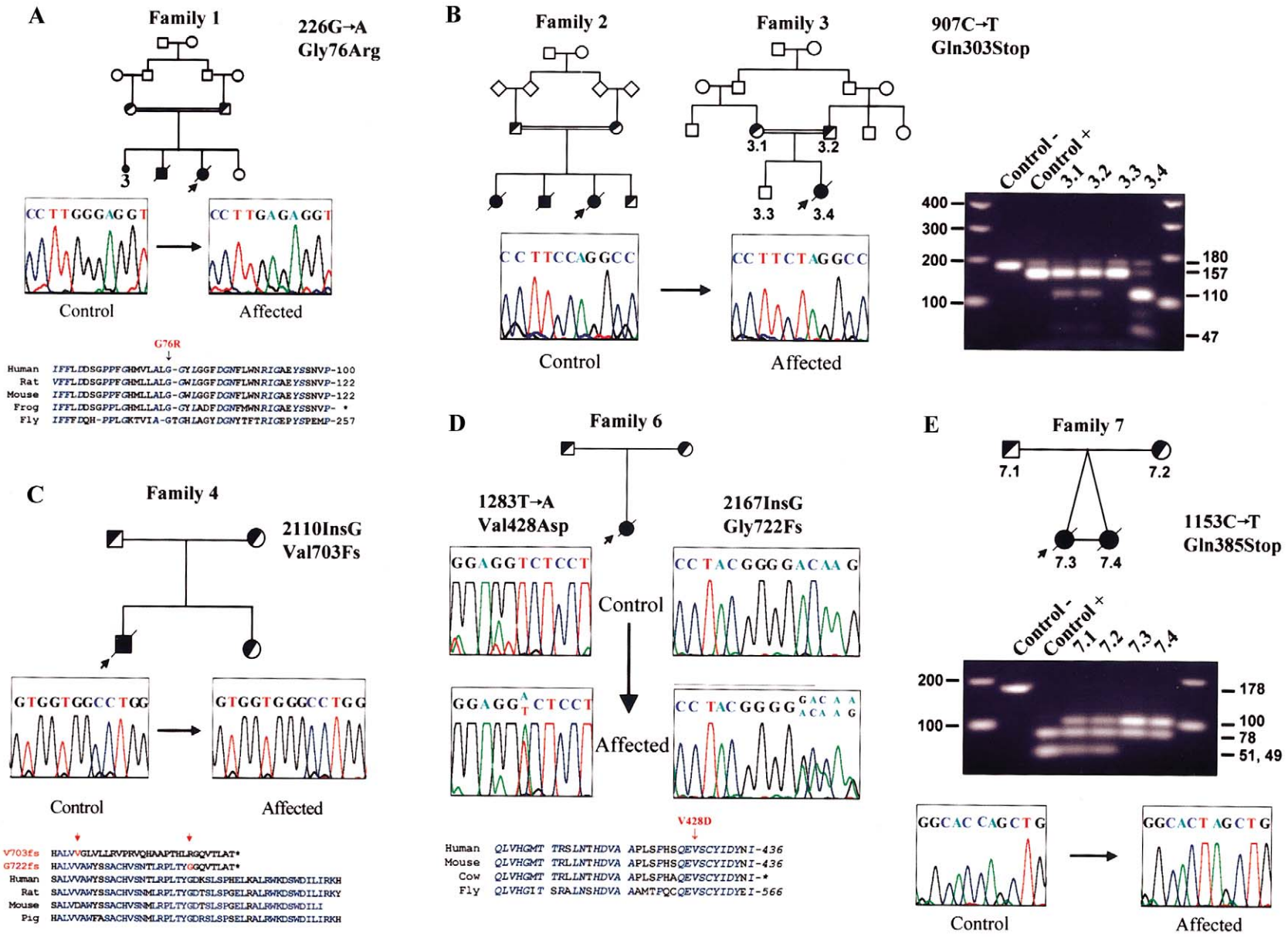
transferase, which has different isoforms as the result of alternative splicing. *POMT1* is ubiquitously expressed with peak levels in adult testis, in skeletal and cardiac muscle, and in fetal brain (Jurado et al. 1999). This expression profile perfectly matches the tissues affected in WWS. In addition to muscle, eye, and brain involvement, male patients with WWS often have testicular defects (Hung et al. 1998).

POMT1 *Mutation Analysis*

Primers were designed for amplification of each of the 20 exons and flanking intron sequences of *POMT1*. Mutation analysis was performed by direct sequencing of the resulting amplicons from patients from the five families with linkage.

Patient 1 has a homozygous transition, 226G→A, predicting a Gly76Arg substitution, in exon 3 (fig. 2*A*). Gly76 is conserved in POMT1 orthologues from different species and is located in the second transmembrane (TM2) domain of the protein (Jurado et al. 1999). The substitution of this hydrophobic amino acid by a basic arginine is likely to disrupt the membrane affinity of this TM2 domain. Patients 2 and 3 are homozygous for a transversion, 907C→T, that creates a Gln303Stop mutation in exon 9 (fig. 2*B*). Patient 4 is homozygous for a frameshift mutation, 2110InsG—predicted to cause a replacement of 44 highly conserved C-terminal amino acids by 26 irrelevant ones after Val703—in exon 20 (fig. 2*C*). No mutation was detected in patient 5. The Mendelian segregation of the mutations was confirmed by direct sequencing of family members. Testing of the mutations in the normal population was performed by SSCP analysis or by restriction-enzyme digestion in the case of Gln303Stop. This mutation generates a new *BfaI* restriction site in exon 9. None of the changes were found in 60 Dutch and 45 Turkish control individuals. During the mutation analysis, several SNPs were identified in the coding region and flanking intronic sequences of the *POMT1* gene. Using these SNPs and dinucleotide markers, we could assign haplotypes for each of the WWS chromosomes. The comparison of WWS haplotypes revealed a shared haplotype and, therefore, a possible common ancestor for the two Turkish families carrying the Gln303Stop mutation (table 1). The other mutations are associated with different haplotypes, indicating a different origin for each of the *POMT1* mutations.

Having established the causative role that *POMT1* gene mutations play in most of the families with WWS and linkage, we sequenced this gene in all available patients with WWS, originating from 10 consanguineous and 15 nonconsanguineous additional families. We found mutations in two of these families. Patient 6 is a compound heterozygote (fig. 2*D*). The first mutation is a transversion, 1283T→A, that causes the replacement of a highly conserved valine by an aspartic acid. The second mutation, 2167InsG, creates a frameshift that is predicted to remove the 25 amino acids following Gly722. Both of the MZ twin girls affected in family 7 are homozygous for a transition, 1153C→T, that gives rise to a stop codon at Gln385. This mutation disrupts a *Pvu*II restriction site used to verify the absence of this mutation in the normal population. Thus, in total, we have found *POMT1* mutations in 6 of the 30 families with WWS; this finding supports the notion, from the genomewide linkage analysis, that WWS is genetically heterogeneous. We next extended our mutational analysis to the other putative O-mannosyltransferases: *POMT2, SDF2,* and *SDF2L1*.


A
Family 1
226G→A
Gly76Arg
3
CC T T GGGAG G T
CC T T GAGAGGT
Control
Affected
G76R
Human IFFLIDSGPPFGHMVLALG-GYLGGFDGNFLWNRIGAEYSSNVP-100
Rat VFFLIDSGPPFGHMLLALG-GWLGGFDGNFLWNRIGAEYSSNVP-122
Mouse IFFLIDSGPPFGHMLLALG-GWLGGFDGNFLWNRIGAEYSSNVP-122
Frog IFFLIDSGPPLGHMLLALG-GYLADFDGNFMWNRIGAEYSSNVP- *
Fly IFFFDQH-PPLGKTVIA-GTGHLAGYDGNYTFTRIGEPYSPEMP-257
B
Family 2
Family 3
907C→T
Gln303Stop
3.1
3.2
3.3
3.4
C C TTCCAGGCC
CC T T CTAGGCC
Control
Affected
Control -
Control +
3.1
3.2
3.3
3.4
400
300
200
100
180
157
110
47
C
Family 4
2110InsG
Val703Fs
GTGGTGGCC T GG
GTGGTGGGCC T GG
Control
Affected
V703fs HALVVGLVLLRVPRVQHAAPTHLRGQVTLAT*
G722fs HALVVAWYSSACHVSNTLRPLTYGGQVTLAT*
Human SALVVAWYSSACHVSNTLRPLTYGDKSLSPHELKALRWKDSWDILIRKH
Rat SALVVAWYSSACHVSNMLRPLTYGDTSLSPGELRALRWKDSWDILIRKY
Mouse SALVDAWYSSACHVSNMLRPLTYGDTSLSPGELRALRWKDSWDILI
Pig HALVVAWFASACHVSNMLRPLTYGDRSLSPSELRALRWKDSWDILIRKH
D
Family 6
1283T→A
Val428Asp
2167InsG
Gly722Fs
GGAGGTCTCCT
CC TAC GGG GACAA G
Control
GGAGG A/T CTCCT
CC TAC GGG G GAC A A / ACA A G
Affected
V428D
Human QLVHGMT TRSLNTHDVA APLSPHSQEVSCYIDYNI-436
Mouse QLVHGMT TRLLNTHDVA APLSPHSQEVSCYIDYNI-436
Cow QLVHGMT TRLLNTHDVA APLSPHAQEVSCYIDYNI-*
Fly QLVHGIT SRALNSHDVA AAMTPQCQEVSCYIDYEI-566
E
Family 7
7.1
7.2
7.3
7.4
1153C→T
Gln385Stop
Control -
Control +
7.1
7.2
7.3
7.4
200
100
178
100
78
51, 49
GGCAC CAG CT G
GGCACT AG CT G
Control
Affected

Direct sequencing did not reveal causative mutations in any of the families with WWS that were sequenced.

Immunological Studies

To investigate whether O-mannosylation is indeed abnormal in patients with WWS who carried *POMT1* mutations, we performed immunohistochemical analysis of the highly O-mannosylated protein α-dystroglycan (Herrmann et al. 2000; Kano et al. 2002) in WWS muscle biopsies (fig. 3). Dystroglycan is synthesized as a precursor that generates two polypeptides by posttranslational cleavage: α- and β-dystroglycan. β-Dystroglycan is attached to the cell membrane, whereas α-dystroglycan associates with it and, through its carbohydrate tails, establishes other interactions with the extracellular matrix proteins, such as laminin-α2, perlecan, and agrin. We have used two different antibodies raised against α-dystroglycan: the monoclonal antibody, VIA4.1, which is thought to recognize the carbohydrate epitope of α-dystroglycan specifically, and the polyclonal antibody, pαDAG, which is raised against the C-terminal end of the protein (Herrmann et al. 2000). VIA4.1 shows a complete lack of staining in muscle biopsies of two independent patients with WWS who carry homozygous *POMT1* mutations. pαDAG immunostudies in the same patients show a marked reduction of α-dystroglycan peptide in many fibers. Misprocessing of the dystroglycan precursor protein is unlikely to be the cause of this reduction, since β-dystroglycan immunoreactivity is normal. These results suggest that, in muscle from these patients with WWS, α-dystroglycan not only is present at reduced levels but also is underglycosylated. The α-dystroglycan O-mannosyl glycans are critical for binding of laminin-α2, which connects the sarcolemma with the extracellular matrix (Brown et al. 1999). Thus, the disruption of this interaction may be sufficient by itself to cause the muscular dystrophy in WWS.

Discussion

In the present article, we have established that mutations in the *POMT1* gene cause WWS. Of 30 patients tested, only 6 (20%) show *POMT1* mutations. The genetic heterogeneity of the disease was already evidenced by the genomewide linkage analysis, which demonstrated the existence of at least three WWS loci, and excluded linkage for the POMT1 locus in several families.

The autosomal recessive inheritance pattern of WWS and the type of mutations identified in patients suggest that the syndrome is caused by a loss-of-function mechanism. POMT1 is not the only human enzyme that is able to catalyze the first step in the O-mannosyl glycan synthesis. Another putative O-mannosyltransferase is POMT2, which shows an expression pattern in adults that overlaps with that from POMT1; both POMT1 and POMT2 show high expression levels in skeletal and cardiac muscle (GenBank accession number AF105020). Despite its similar function and expression profile, POMT2 is apparently unable to compensate for POMT1 loss-of-function mutations. This could be due to differences in their expression patterns during embryonic development or because of a differential arrangement of proteins that are modified by these two mannosyltranferases. We have not found any *POMT2* mutation in 30 unrelated patients with WWS.

The disruptive effect of the *POMT1* mutations is likely mediated through the reduced or absent O-mannosylation of target proteins, which affects their normal function in development. The implication, in MEB and WWS, of two glycosyltransferases involved in O-mannose glycan synthesis clearly shows the relevance of O-mannosylation for eye, muscle, and brain development. All patients carrying *POMT1* mutations show the typical features of WWS (fig. 1), as do other patients in whom no mutations were detected. A previous reported linkage analysis (Cormand et al. 2001), together with the genomewide linkage data from the present study, allows us to exclude the MEB and FCMD loci in 8 of 10 families

Figure 2 *POMT1* gene mutations in families with WWS. For each of the families with POMT1 mutations, the pedigrees are shown, as well as two chromatograms, corresponding to a patient and a control DNA from the normal population. Patients for whom sequencing results are shown are marked by an arrow. *A,* Family 1. The patient is homozygous for a 226G→A mutation (Gly76Arg). A multiple-sequence alignment of the POMT1 peptide sequence from different species around glycine 76 is shown. * = not known. *B,* Families 2 and 3, carrying the same homozygous mutation, 907C→T (Gln303Stop). This mutation creates a new *Bfa*I site, which was used to analyze the segregation of this mutation in family 3. The 180-bp amplicon is digested into 23- and 157-bp products for the wild-type allele, and the mutant allele is digested into products of 110, 47, and 23 bp. Undigested (−) and digested (+) products were loaded for the control. *C,* Sequence analysis of family 4 reveals homozygosity of 2110InsG (Val703fs). The bottom portion shows the comparison of the C-terminal peptide sequence from different species, as well as the predicted protein encoded by the 2110InsG and 2167InsG (Gly722fs) alleles. The mutation is marked by an arrow. * = predicted stop codon. *D,* Patient with WWS from family 6, who is a compound heterozygote for the *POMT1* mutations 1283T→A (Val428Asp) and 2167InsG (Gly722fs). * = not known. *E,* MZ twins from family 7, who are homozygous for the 1153C→T (Gln385Stop) mutation. This mutation disrupts a *Pvu*II site, which was used to study the segregation in the family. The 178-bp amplicon is digested into 78- and 100-bp products for the wild-type allele, and into 49-, 51-, and 78-bp products for the mutant allele.

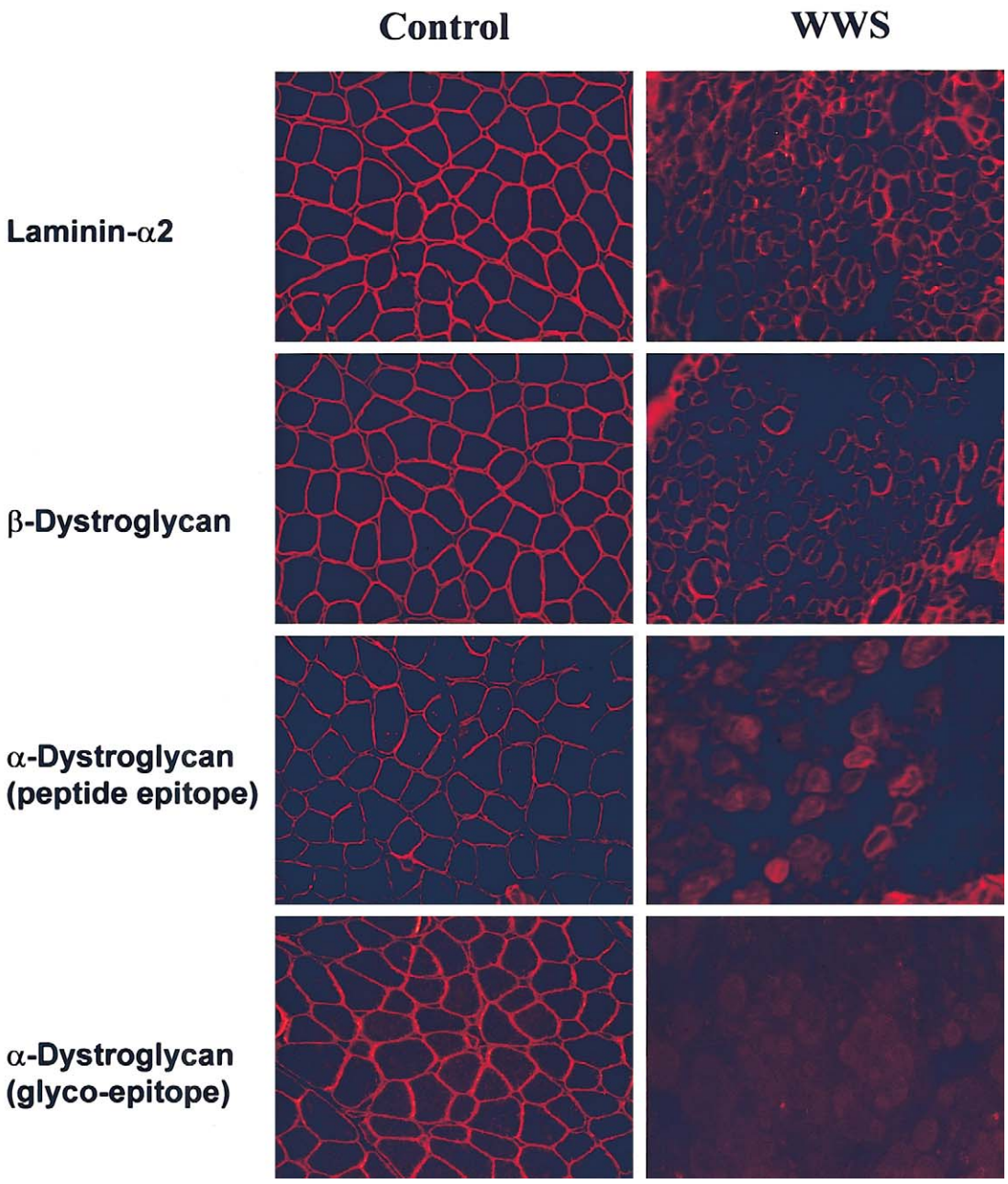


Figure 3 Immunofluorescence staining of skeletal muscle from a control individual and from patients with WWS from families 2 and 4. Note the normal expression of laminin-α2 and β-dystroglycan in WWS. Variable staining intensities were observed with anti-α-dystroglycan antibody specific for the C-terminal peptide sequence, with most muscle fibers showing a marked reduction in α-dystroglycan staining. No α-dystroglycan staining was observed in patient muscle by using an antibody thought to recognize the O-glycan motif.

with WWS that were investigated. Given that the WWS and MEB genes are both involved in O-mannosylation and given the genetic heterogeneity shown for these related syndromes, it is very likely that other proteins involved in this type of glycosylation underlie the as-yet-unexplained patients.

All of the O-mannose–linked glycans identified to date contain the motif Galβ1 4GlcNAcβ1 2Man-O-Ser/Thr (O-mannose–linked core), to which other sugar residues can be sequentially added (fig. 4). Most of the resultant variants are brain specific, and only one such motif has been identified in both brain and muscle (Siaα2 3Galβ1 4GlcNAcβ1 2Man-O-Ser/Thr; fig. 4). Both POMGnT1 and POMT1 are involved in the synthesis of the O-

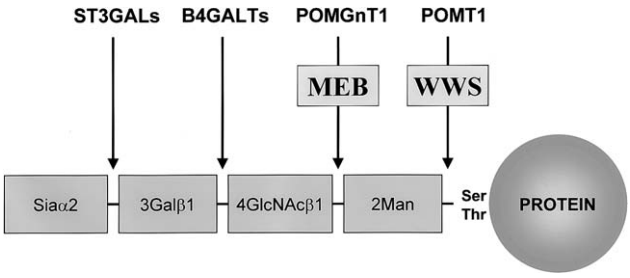


Figure 4 Representation of the only O-mannose–linked glycan motif found in brain, muscle, and peripheral nerve. The big circle represents the protein, to which several sugar residues are added in a sequential manner by the catalytic activity of specific glycosyltransferases. POMT1 catalyzes the addition of the first residue (mannose), whereas POMGnT1 catalyzes the second step, the linkage of N-acetylglucosamine in β-1,2. B4GALTs (Amado et al. 1999) and ST3GALs (Tsuji 1996) are the families of enzymes that are able to catalyze the third and fourth steps, respectively, of this O-glycan synthesis. The specific enzymes that catalyze these steps have not been assigned yet.

mannose–linked core. Since POMT1 catalyzes the first step and POMGnT1 catalyzes the second step in O-mannose glycosylation (fig. 4), one might expect the phenotype associated with *POMT1* mutations to be more severe than that associated with *POMGnT1* mutations. One might therefore speculate that mutation of glycosyltransferases that catalyze subsequent steps gives rise to a phenotype that is less severe than MEB/WWS. However, the level of redundancy at each of the glycosylation steps is not known, and, therefore, it can also be envisaged that disruption of an enzyme that catalyzes a later step will give rise to an equally severe phenotype.

The O-glycans attached to α-dystroglycan play a role in molecular recognition (Brown et al. 1999). The immunochemistry results shown in the present article illustrate a secondary reduction in the protein levels of α-dystroglycan. Secondary reductions of dystrophin-glycoprotein complex (DGC) components, in which α-dystroglycan is included, have been described in other muscular dystrophies (Ohlendieck and Campbell 1991; Barresi et al. 1997). The stability of each component of the DGC depends on the correct interaction with its neighboring proteins, and the absence of one of the components of the DGC often causes a reduction of its interacting proteins as well. Most likely the depletion of the O-mannosyl glycans prevents the normal interaction between α-dystroglycan and laminin-α2, and this disturbance reduces the α-dystroglycan stability. In agreement with this, several patients with WWS who have been described in the literature showed reduced levels of the α-dystroglycan–interacting muscle proteins laminin-α2 (Kanoff et al. 1998), laminin-β2, and adhalin (Wewer et al. 1995).

Hypoglycosylation of α-dystroglycan may well be sufficient to explain the muscular dystrophy that is observed in patients with WWS. O-glycans are required for the attachment of α-dystroglycan to the extracellular matrix, which is crucial for the correct contraction of the muscle. Apart from WWS, MEB, and FCMD, mutations in two other glycosyltransferases—Large, in mice, and FKRP, in human—cause hypoglycosylation of α-dystroglycan and muscular dystrophy (Brockington et al. 2001*a;* Grewal et al. 2001). FKRP and Large mutants do not present severe brain symptoms.

The brain and eye phenotypes in WWS and MEB probably involve defective glycosylation of proteins other than α-dystroglycan, since chimeric mice deficient in dystroglycan develop muscular dystrophy but do not display a brain or eye phenotype (Cote et al. 1999). In brain, other possible O-mannosylation targets that may be responsible for the WWS symptoms include reelin and tenascin-J1 (Lochter et al. 1991; Hirotsune et al. 1995). Both proteins are diffusible factors that show a gradient distribution in brain development. Reelin is a glycoprotein secreted by Cajal-Retzius cells and other pioneer neurons and is implicated in lissencephaly with cerebellar hypoplasia (Hong et al. 2000). Tenascin-J1 is a highly O-mannosylated glycoprotein that shows repellent activity on neurite extension in neuroblast culture (Wing et al. 1992).

The neuronal overmigration and the invasion of the subarachnoid space by the migrating neurons in the WWS neocortex (figs. 1*E* and 1*F*) resembles the situation present in laminin-α6 and myristoylated alanine-rich protein C kinase substrate (MARCKS) knockout mice (Stumpo et al. 1995; Georges-Labouesse et al. 1998). In the MARCKS knockout, it has been hypothesized that the disturbance of structural components of the basal lamina is responsible for the neuronal overmigration, whereas, in laminin-α6, the recognition process between the matrix and the migrating neurons is disrupted. Similarly failed interactions with extracellular matrix components may underlie the severe neuronal overmigration that causes cobblestone lissencephaly in WWS. It is interesting that, whereas integrin-

α3 and integrin-α6 have been implicated in neocortex lamination, integrin-α5 and integrin-α7 are involved in muscle development (Mayer et al. 1997; Georges-Labouesse et al. 1998; Taverna et al. 1998; Anton et al. 1999). It is tempting to speculate that the α-integrins may also be targets for O-mannosylation.

Note added in proof.—While the present article was in press, two papers were published that are relevant to the pathogenesis of WWS. The first article (Michele et al. 2002) shows that, in patients with MEB and FCMD, α-dystroglycan is hypoglycosylated and no longer interacts with proteins from the extracellular matrix. In the second article (Moore et al. 2002), it is demonstrated that the brain-specific disruption of α-dystroglycan during mouse embryonic development causes neuronal overmigration and fusion of cerebral hemispheres, resembling WWS brain abnormalities. These papers indicate that the disrupted glycosylation of α-dystroglycan makes a major contribution to the CNS abnormalities in WWS.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *POMT2* [accession number AF105020])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for WWS [MIM 236670], MEB [MIM 253280], and FCMD [MIM 253800])

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