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Targeted Disruption of the Mouse Phosphomannomutase 2 Gene Causes Early Embryonic Lethality

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Mutations in the cytosolic enzyme phosphomannomutase 2 (PMM2), which catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate, cause the most common form of congenital disorders of glycosylation, termed CDG-Ia. It is an inherited multisystemic disease with severe neurological impairment. To study the pathophysiology of CDG-Ia and to investigate possible therapeutic approaches, we generated a mouse model for CDG-Ia by targeted disruption of the *Pmm2* gene. Heterozygous mutant mice appeared normal in development, gross anatomy, and fertility. In contrast, embryos homozygous for the *Pmm2*-null allele were recovered in embryonic development at days 2.5 to 3.5. These results indicate that Pmm2 is essential for early development of mice. Mating experiments of heterozygous mice with wild-type mice could further show that transmission of the female *Pmm2*-null allele is impaired.

The attachment of oligosaccharide moieties onto newly synthesized proteins presents one of the most widespread forms of co- and posttranslational modifications that are found in animals, plants, and bacteria. Glycoproteins are most abundant in extracellular fluids and matrices but are also found in subcellular organelles and in cellular membranes. The oligosaccharide moieties of glycoproteins affect their folding, their transport, and their biological activity and stability. The complex process of protein glycosylation requires more than a hundred glycosyltransferases, glycosidases, and transport proteins. Oligosaccharide moieties are connected to glycoproteins predominantly by either N-glycosidic linkages, in which glycans are linked to amino groups of asparagine side chains, or by O-glycosidic linkage, where the glycans are bound to hydroxyl groups of serine or threonine side chains (4).

Inborn errors in protein glycosylation in man lead to congenital disorders of glycosylation (CDG), a rapidly growing group of (thus far) 18 monogenic inherited diseases that present most often with a severe neurological phenotype (9, 16, 17, 29). CDG-Ia (OMIM 212065) represents by far the most widespread form of these diseases, affecting about 500 diagnosed patients worldwide. The multisystemic disease presents with hypotonia, psychomotor retardation, peripheral neuropathy, and cerebellar atrophy (8). CDG-Ia is caused by mutations in the gene encoding PMM2 (18, 28), the enzyme which catalyzes in the cytosol the conversion of mannose-6-phosphate to mannose-1-phosphate. Mannose-1-phosphate is further converted to GDP-mannose, the donor substrate for the addition of mannose residues in the biosynthesis of dolichol-linked oligosaccharides in N glycosylation. The reduced activity of PMM2 in CDG-Ia patients leads to a decreased amount of GDP-mannose and dolichol-phosphate-mannose, which ends up in shortened lipid-linked oligosaccharides, leading to a reduced transfer of these oligosaccharides to nascent glycoproteins by the oligosaccharyl transferase complex (12). Although the glycosylation deficiency can efficiently be corrected in cultured skin fibroblasts from CDG-Ia patients by mannose addition (21, 24) or glucose deprivation (13), the therapeutic effects could not be achieved in CDG-Ia patients themselves (20). A better understanding of the pathophysiology of CDG-Ia is needed to enable the development of an effective therapy for this disorder. Due to the fact that human PMM2 and mouse Pmm2 show an identity of ca. 90% at the protein level (5), we investigated the disruption of the open reading frame of *Pmm2* in the mouse.

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MATERIALS AND METHODS

Isolation of a genomic clone and construction of a gene targeting vector. A C57BL/6J mouse BAC library (5) was screened with a full-length mouse *Pmm2* cDNA (see discussion of reverse transcription-PCR [RT-PCR] analysis below). The isolated mouse BAC clone contained exons two to four of the eight exons of the mouse *Pmm2* gene, as well as several kilobase pairs flanking the 5′ and 3′ regions. DNA sequences analyses revealed complete sequence identity of exon sequences to the nucleotide sequence of the mouse *Pmm2* cDNA used for screening (reference 5 and data not shown). For construction of a target vector, a 9.8-kbp BamHI DNA restriction fragment of *Pmm2* gene covering 4.4 kbp 5′ upstream of exon 2 and 1.9 kbp 3′ downstream of exon 4 was subcloned into the plasmid vector pBluescript SK II(+) (Stratagene), generating pB-Pmm2-BamHI (Fig. 1). This plasmid was digested by the restriction enzyme HindIII. The isolated 6.5-kbp HindIII-fragment, containing exons 2 to 4 (Fig. 1), was cloned into pBluescript SK II(+), in which the BamHI restriction site in the multiple cloning site of this vector has previously been deleted by using a QuickSite mutagenesis kit (Stratagene) with the primers BamHI-del-Fw (5′-GCCCGGG GCATCCACTAGTTCTAGA-3′) and BamHI-del-Rev (5′-TCTAGAACTAGT GGATGCCCCGGGC-3′), leading to plasmid pB-Pmm2-HindIII. Using the QuickSite mutagenesis kit with the primers BamHI-Fw (5′-GCAGCCCGGGG GATCCGAACAAAC-3′) and BamHI-Rev (5′-GTTTGTTCGGATCCCCCGG GCTGC-3′), a BamHI restriction site was generated in exon 2 of the gene. The neomycin expression cassette from pMC1neopA (Stratagene) was inserted as a BamHI DNA restriction fragment into the newly generated restriction site in exon 2, leading to pB-Pmm2-KO. The insertion of the neo cassette introduced a

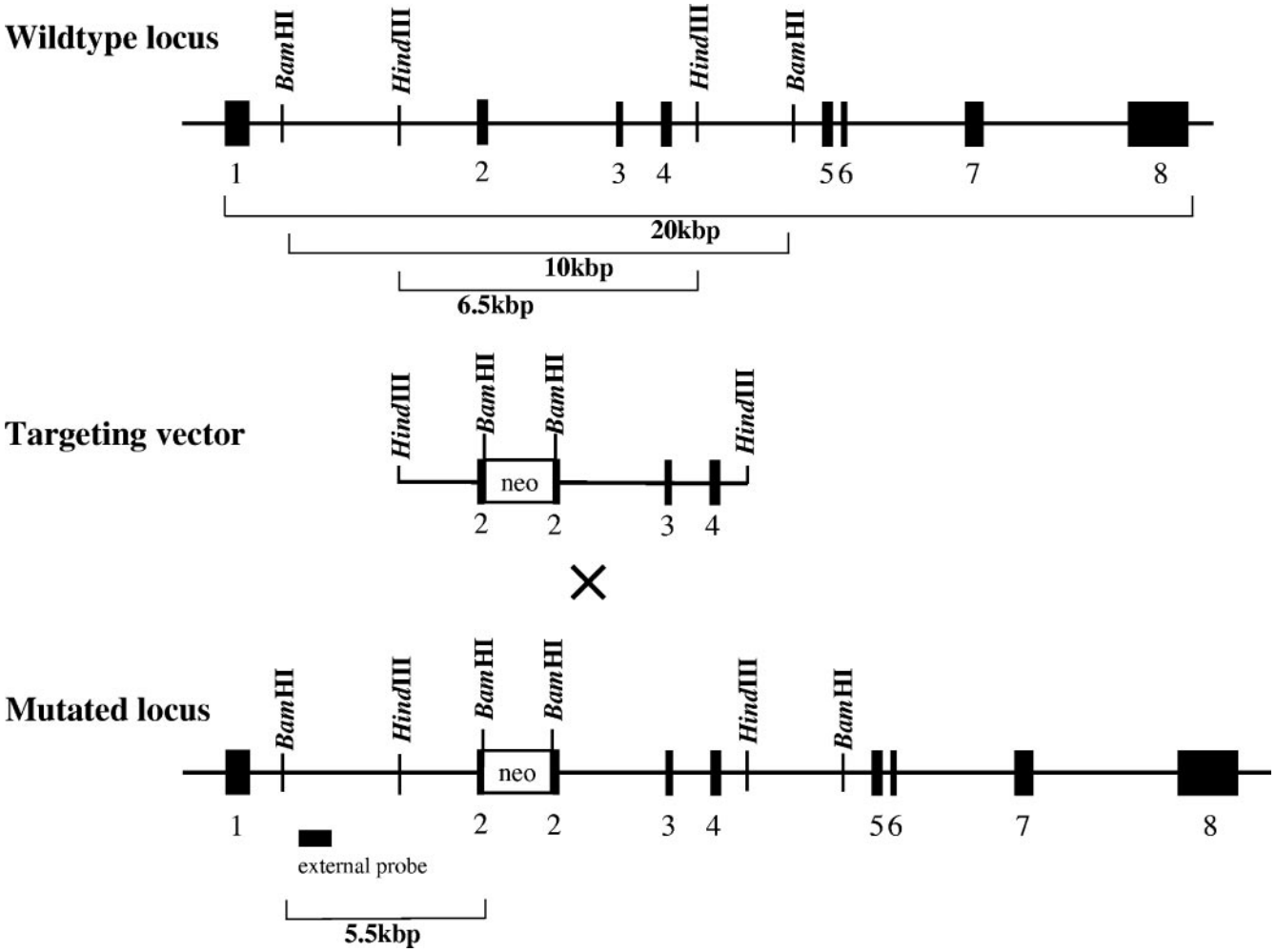

FIG. 1. Genomic organization of mouse *Pmm2* gene and targeting construct. Exons are depicted by filled boxes. The probe location for Southern blotting is shown. The insertion of a neomycin resistance cassette into exon 2 of mouse *Pmm2* leads to a 7.5-kbp HindIII fragment that was used as a targeting vector for the electroporation of mouse ES cells.

frameshift into the open reading frame of the *Pmm2* gene, generating a null allele of mouse *Pmm2*.

Selection of targeted ES cells and generation of mutant mice. The recombination construct (pB-Pmm2-KO; see Fig. 1) was digested with restriction enzyme HindIII. The 7.5-kbp *Pmm2* fragment containing the neo cassette was gel extracted. A total of 20 μg of this fragment was introduced into mouse embryonic stem (ES) cell line E14-1 by electroporation (7). G418-resistant colonies were screened by Southern blot analysis of genomic DNA digested with BamHI and probed with 440 bp of intron 1 as an external probe (Fig. 2A), amplified with primers TL-5′-Sonde-F1 and TL-5′-Sonde-R1. ES cell clones showing a 5.5-kbp BamHI fragment in Southern blot analysis were microinjected into mouse blastocysts of C57BL/6J mice. The resulting male chimeras were mated with C57BL/6J female mice to obtain heterozygous F_1 offspring. F_1 mice were genotyped for the introduced *Pmm2* gene mutation by Southern blot analysis as described above. The mice were kept on a strict 12-h light-dark cycle in a conventional animal facility at the Zentrale Tierexperimentelle Einrichtung, Uniklinikum Goettingen (Gottingen, Germany). Heterozygous F_1 littermates were mated to obtain homozygous F_2 mice.

RT-PCR analysis. Total RNA was extracted from the tails of heterozygous and wild-type animals by using the RNeasy kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. First-strand synthesis was carried out according to the guidelines for the Omniscript RT kit (QIAGEN), using 1 μg of total RNA and the primer mPmm2-R1 (5′-GGTGGGTAACTCTGGGTGGC-3′). PCR was done with the HotStarTaq-Polymerase kit (QIAGEN) according to the manufacturer's instructions with the primers mPmm2-R1 and mPmm2-F1 (5′-GGAAGTTCCGGGTAGAGTTCC-3′) in a volume of 50 μl with 5 μl of the reverse transcriptase reaction as template for each. Nested PCR was done with the primers mPmm2-F2 (5′-GAGTTCCTGGTGCGGTCGAG-3′) and mPmm2-R2 (5′-CAGTGTTGTTGGTCAGTCCGG-3′). We used 0.1 μl from the first PCR as a template for the nested PCR. The PCR products were analyzed on 1% agarose gels, gel extracted, and subsequently sequenced using the primers mPmm2-F2 and mPmm2-R2.

Northern blot analysis. A total of 10 μg of total RNA isolated from heterozygous and wild-type mice (QIAGEN RNA kit) was used per lane in Northern blot analysis. RNA was supplemented with 2xRNA loading buffer (MBI) and loaded on a 1.5% agarose gel. Further procedures were performed as described previously (14). The complete mouse cDNA was used as a probe.

Western blot analysis. Portions (100 μg) of protein isolated from the tails of heterozygous and wild-type animals were electrophoresed under denaturing conditions on 12.5% sodium dodecyl sulfate gels and blotted on a nitrocellulose membrane (Sartorius, Gottingen, Germany) and blocked with 5% nonfat milk. The antibody Pmm2 (2) was used at a dilution of 1:2,000 in blocking buffer (Blotto; Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk). For detection, a horseradish peroxidase-coupled secondary antibody was used, followed by enhanced chemiluminescence (Amersham, United Kingdom).

Analysis of embryos and blastocysts. Embryos were harvested from timed matings of heterozygous intercrosses. Genotyping of Pmm2 embryos from embryonic days 15.5, 12.5, and 9.5 was performed by Southern blotting. Genomic DNA was isolated from fetal membranes or whole embryos. For genotyping of blastocysts (embryonic day 3.5), heterozygous female Pmm2 animals were su-

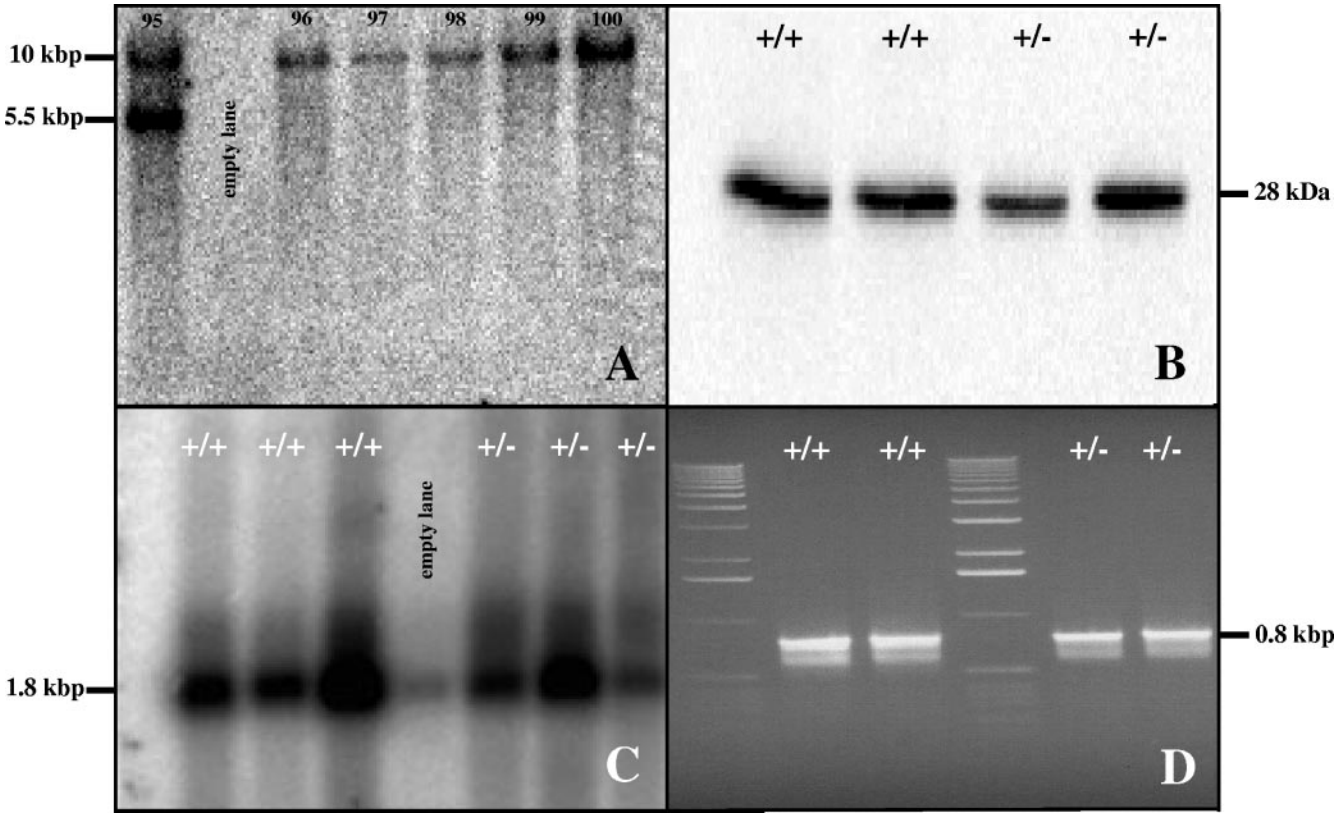

FIG. 2. (A) Southern blot analysis of ES cell clones. Genomic DNA isolated from mutated ES cell clone 95 (left) and wild-type clones 96 to 100 (right) was digested with restriction endonuclease BamHI and analyzed by Southern blotting leading either to a 5.5-kbp BamHI fragment in the case of the recombined *Pmm2* allele (clone 95) or to a 10-kbp BamHI fragment in the case of the wild-type allele (clones 96 to 100). (B) Mouse tail immunoblot analysis. Immunoblot analysis of the molecular weight of two heterozygous and two control mice was done with an antibody to mouse Pmm2. For detection, a horseradish peroxidase-coupled secondary antibody was used. (C) Northern blot analysis of *Pmm2* expression. Total mRNA isolated from mouse tails of three heterozygous and three control mice was analyzed by Northern blotting using the full-length mouse *Pmm2* cDNA as a probe. (D) RT-PCR analysis of *Pmm2* transcripts. Analysis of *Pmm2* transcripts of two heterozygous and two control mice by RT-PCR.

perovulated with 10 IU of pregnant mare serum and 24 h later with 10 IU of human chorionic gonadotropin. After 48 h, female animals were mated to heterozygous male mice. Blastocysts were flushed from the uteri at embryonic day 3.5 postcoitum (3) using M2 buffer (Sigma). Blastocysts were put in 5 μl of phosphate-buffered saline (PAA) and frozen for 10 min on dry ice. After incubation for 10 min at 95°C, 20 μg of proteinase K (in 2 μl) was added, followed by incubation for 35 min at 56°C. Digestion was stopped by incubation at 95°C for 8 min. The whole volume was used for PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation for 30 s at 94°C, primer annealing of primer 4-1 (5′-CCTCAGCATTAGTCGTGGGC-3′) and primer 37-1 (5′-GCTTGCTG ATTCAGGGTAGGC-3′) for 30 s at 55°C, and elongation for 3 min at 72°C. Nested PCR was performed under the same conditions, using 1 μl of the first PCR as a template and the primers 4-2 (5′-CAGTTGCTCTTGCAGTTTCTC AAATCTGACCC-3′) and 37-2 (5′-GCTTGCTGATTCAGGGTAGGC-3′). PCR fragments were analyzed by agarose gel electrophoresis.

Genotype analysis of embryonic day 2.5 fertilized eggs. Heterozygous female Pmm2 mice were superovulated and mated to heterozygous male mice as described above. Fertilized eggs were isolated from uteri 2.5 days postcoitum. Genomic DNA was prepared as described above and used completely for PCR analysis under the same conditions as described above with primers 4-1 and 37-1. After the run the whole sample was extracted using the MiniElute Kit (QIAGEN). PCR fragments were resolved in 10 μl of 10 mM Tris-HCl (pH 8.5) and used completely as a template for a second PCR, followed by a new extraction as stated above. A third PCR using the complete template from the extraction of the second PCR was performed with the primers 4-2 and 37-2. PCR products were analyzed on a 1.5% agarose gel.

Test of transmission of the *Pmm2*-null allele. Heterozygous male mice were mated with wild-type female mice, and heterozygous female mice were mated with wild-type male mice. Littermates of both matings were analyzed by Southern blot analysis for their genotype.

RESULTS

A mouse genomic DNA clone covering 10 kbp of the *Pmm2* gene, including exons 2 to 4, was isolated from a C57BL/6J mouse BAC library. The target construct pB-Pmm2-KO (Fig. 1) was used for the electroporation of mouse ES cells (E14-1). The open reading frame of the gene was interrupted in exon 2 by the insertion of a neomycin resistance cassette, leading to a null allele of *Pmm2* (Fig. 1). G418-resistant colonies were analyzed by Southern blotting. In 3 of 145 independent clones tested, an additional BamHI restriction side was detected with an external probe, indicating a homologous recombination event in one of the *Pmm2* alleles (Fig. 2A). The targeted ES cell clone 95 was microinjected into C57BL/6J blastocysts. The resulting male chimeras with a chimerism of 20, 30, 50, and 60% were mated with C57BL/6 female mice. Only the 50% chimeric mice showed germ line transmission and generated heterozygous offspring. Heterozygous offspring showed no differences in morphological phenotype compared to the wild-type animals. Genotyping of 220 littermates from heterozygous

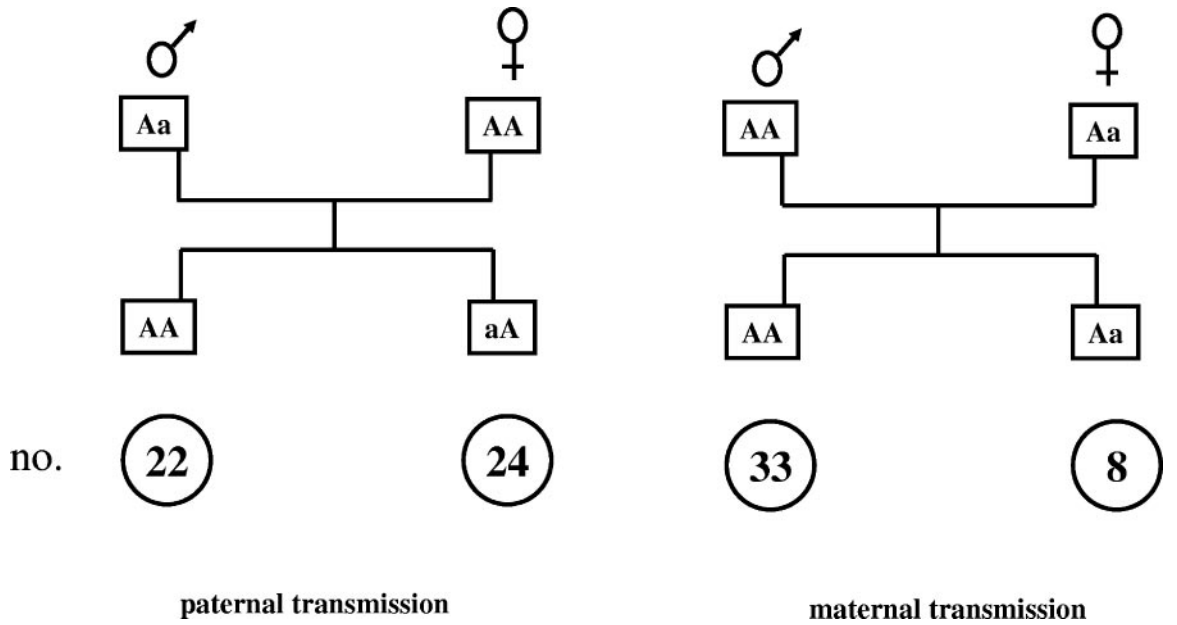

FIG. 3. Difference between paternal and maternal transmission of the *Pmm2*-null allele. Intercrosses from heterozygous Pmm2 mice with wild-type mice were analyzed for their genotype. Symbols and abbreviations: ♂, male; ♀, female, AA, wild type, Aa, heterozygous; no., number of heterozygous or wild-type offspring.

crosses revealed frequencies of 52% for wild-type mice and 48% for heterozygous mutant mice. Mice homozygous for the mutation could not be detected. This suggests that a complete loss of Pmm2 activity is lethal to the embryo. After mating of male mice heterozygous (+/−) for the *Pmm2*-null allele with wild-type (+/+) female mice, we observed 22 homozygous wild-type and 24 heterozygous animals (Fig. 3), indicating that the relation is nearly 1:1 for the paternal transmission. In contrast, the mating of female mice (+/−) heterozygous for the *Pmm2*-null allele with wild-type (+/+) male mice led to 33 homozygous wild-type and 8 heterozygous mice, resulting in a ratio of 4:1 (Fig. 3). To test for the expression of a Pmm2/neomycin resistance hybrid protein at RNA and protein levels, RT-PCR and Northern and Western blot analyses were performed with material from heterozygous and wild-type animals. RT-PCR was performed with primers specific for mouse *Pmm2* cDNA. Both wild-type and heterozygous animals showed only the expected 800-bp fragment (Fig. 2D), specific for wild-type *Pmm2* cDNA. Northern blot analysis revealed only a 1.8-kbp *Pmm2*-specific RNA hybridizing in total RNA from wild-type and heterozygous animals (Fig. 2C). Western blot analysis of three wild-type and three heterozygous mice revealed no differences in Pmm2 protein size (Fig. 2B). Embryos of embryonic days 15.5, 12.5, and 9.5, as well as blastocysts of embryonic day 3.5, were tested for their genotype (Table 1), showing only wild-type or heterozygous genotypes. In the case of the isolated 33 fertilized eggs of embryonic day 2.5, 16 were identified as wild type, 14 had a heterozygous genotype, and 3 were homozygous for the Pmm2 knockout.

TABLE 1. Genotype analysis of litters of heterozygous Pmm2 intercrosses[a]

Stage	No. of mice			
	Total	Wild type (+/+)	Heterozygous (+/−)	Homozygous (−/−)
F_2 to F_6 offspring	220	114	106	0
Embryonic day 15.5	9	4	5	0
Embryonic day 12.5	7	3	4	0
Embryonic day 9.5	9	4	5	0
Embryonic day 3.5	13	7	6	0
Embryonic day 2.5	33	16	14	3

[a] The genotypes of embryos from different stages (embryonic days 2.5, 3.5, 9.5, 12.5, and 15.6), as well as newborn mice, were determined.

DISCUSSION

In the present study, we demonstrate that mouse Pmm2 is essential for early embryonic development. Pmm2-null embryos were recovered from embryonic day 2.5 to embryonic day 3.5. Our results indicate that loss of Pmm2 activity either leads to implantation failure or to embryonic death prior to implantation. Genotype analysis of 220 littermates from intercrosses of heterozygous animals revealed a ratio of nearly 1:1 for wild-type to heterozygous mice. No homozygous-deficient animals have been detected. Staying in contrast to the expected Mendelian frequency of 25% wild-type to 50% heterozygous to 25% homozygous deficient animals if heterozygous animals are crossed, our data lead to the assumption that a complete knockout of Pmm2 is not compatible with life. This is consistent with the situation in CDG-Ia patients, who often show a compound heterozygosity for two mutations, at least one of which has a residual activity. The most common mutation described in CDG-Ia patients, R141H, has never been identified in the homozygous state (19, 25). Analysis of the recombinant PMM2 protein with the R141H mutation revealed no rest activity of the mutated protein (11, 22). Since a change in a single amino acid is sufficient for the complete loss of PMM2

activity, the same would have to be expected for a complete knockout of PMM2. The early mortality in Pmm2 knockout mice indicates that no other enzyme can rescue a mouse deficient for Pmm2. This also includes Pmm1 (OMIM 601786) (2a), the predicted isoenzyme of Pmm2 (23). Although both enzymes show a 67% identity to each other, catalyze the same biochemical reaction, and are expressed in the same organs, with the main expression for Pmm1 in brain and for Pmm2 in liver (2, 3, 23), Pmm1 activity seems not to be sufficient for the development of Pmm2-null embryos. Studies of *Pmm2* transcripts by RT-PCR of control and heterozygous mice only showed *Pmm2* cDNA of the expected wild-type size when amplified with primers specific for mouse *Pmm2* cDNA. Northern blot analysis with total RNA isolated from control and heterozygous animals with a *Pmm2*-specific probe showed as well only one band with a size corresponding to the length of wild-type *Pmm2* mRNA (5). Western blotting also demonstrated that only Pmm2 proteins with an expected size of the wild-type protein of 27 kDa were synthesized, with a slight decrease in the amount of Pmm2 protein in heterozygous Pmm2-deficient mice. We postulate, therefore, that no hybrid Pmm2/neomycin resistance product was produced as a result of unstable RNA and nonsense-mediated decay in the case of the targeted allele. To investigate the day of embryonic lethality of Pmm2 knockout mice, embryos of heterozygous intercrosses of different days (E15.5, E12.5, and E9.5), blastocysts of embryonic day E3.5, as well as fertilized eggs of embryonic day E2.5, were isolated and subjected to genotype analysis. Between embryonic days E3.5 and E15.5 a ratio of wild-type to heterozygous genotypes of nearly 1:1 could be detected, which is comparable to the ratio of 52% for wild-type and of 48% for heterozygous genotypes of our postnatal analyzed mice. On embryonic day 2.5, 3 of 33 fertilized eggs were identified as homozygous mutants, which is equivalent to 9%, where 48% of the embryos were wild type and 42% heterozygous for the null allele. The reduction of the homozygous mutant genotype implies that some of the $-/-$ mutants disappeared before the first morphological event of differentiation. Kitamura et al. suggest that glycoproteins are involved in the intercellular recognition and adhesion between the embryo and endometrial epithelium of the uterus (10).

The lethality caused by inactivation of Pmm2 occurs around embryonic day E2.5, a finding which corresponds to the onset of transcription and the increase in translational activity in the major phase of zygotic genome activation. Oocyte maturation also initiates the destruction of maternal RNA. This continues through the two-cell stage, by which time about 90% of the maternal mRNAs is degraded (27). The early embryonic lethality of the Pmm2 knockout mice therefore indicates the important role of glycoproteins in the beginning of development. This result corresponds to data that describe early embryonic lethality in a mouse model with a deficiency of *N*-acetylglucosamine-1-phosphotransferase, the enzyme that catalyzes the addition of the first *N*-acetylglucosamine residue onto the lipid carrier dolichol at the beginning of dolichol-linked oligosaccharide biosynthesis (15).

According to the Mendelian rule, crosses of heterozygous PMM2 knockout animals should result in 25% wild-type, 50% heterozygous, and 25% homozygous animals. A ratio of 52% wild-type to 48% heterozygous animals can be expected if the transmission of the paternal or maternal null allele is impaired. Experiments in which heterozygous female mice were mated with wild-type male mice could show that the male null allele is transmitted to 48% of the litter, where a ratio of nearly 4:1 wild-type to heterozygous animals has been identified in the case of crossing heterozygous female to wild-type male mice. A total of 80% of the offspring were wild type and 20% showed the heterozygous genotype, indicating that transmission of the female null allele is severely impaired. This result might be explained by hypo-N and/or O glycosylation of available glycosylation sides of glycoproteins such as ZP1, ZP2, and ZP3 of the zona pellucida, which are normally highly glycosylated (1). The zona pellucida is a transparent envelope surrounding the mammalian oocyte and plays a role in sperm-egg interactions. This observation is at odds with the situation of the R141H and other mutations in humans, where there is a preferential transmission of the disease allele for both parents (26). Although it has not yet been determined completely whether distinct glycans are necessary for the attachment of the sperm to the oocyte or whether the supramolecular structure of the zona matrix binds the male sperm (6), we can postulate that in the case of female Pmm2 knockout mice the fertilization and/or the oogenesis is affected because of a glycosylation defect in oocytes carrying a *Pmm2*-null allele.

Based on the present study, the activity of Pmm2 is essential for the early development of mouse embryos. Mating experiments of heterozygous mice with wild-type mice could further show that transmission of the female *Pmm2*-null allele is impaired. To overcome the early embryonic lethality, we are currently generating mice that are expected to express a residual activity for Pmm2 similar to human CDG-Ia patients. These mice will be compound heterozygous for the two most frequent mutations observed in CDG-Ia patients: F119L and R141H. This genotype is associated with a very severe form of the disease. Since F119L and R141H are conserved between humans and mice, the animal model is expected to mimic the human disorder. Such a mouse model would allow investigations on the complex pathophysiology of the disease, as well as studies on new therapeutic strategies.

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