Ablation of Mouse Phosphomannomannose Isomerase (Mpi) Causes Mannose 6-Phosphate Accumulation, Toxicity, and Embryonic Lethality*\(^{S}\)

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**Mpi** encodes phosphomannomannose isomerase, which interconverts fructose 6-phosphate and mannose 6-phosphate (Man-6-P), used for glycoconjugate biosynthesis. **Mpi** mutations in humans impair protein glycosylation causing congenital disorder of glycosylation type Ib (CDG-Ib), but oral mannose supplements normalize glycosylation. To establish a mannose-responsive mouse model for CDG-Ib, we ablated **Mpi** and provided dams with mannose to rescue the anticipated defective glycosylation. Surprisingly, although glycosylation was normal, **Mpi**\(^{-/-}\) embryos died around E11.5. Mannose supplementation even hastened their death, suggesting that mannose was toxic. **Mpi**\(^{-/-}\) embryos showed growth retardation and placental hyperplasia. More than 90% of **Mpi**\(^{-/-}\) embryos failed to form yolk sac vasculature, and 35% failed chorioallantoic fusion. We generated primary embryonic fibroblasts to investigate the mechanisms leading to embryonic lethality and found that mannose caused a concentration- and time-dependent accumulation of Man-6-P in **Mpi**\(^{-/-}\) fibroblasts. In parallel, ATP decreased by more than 70% after 24 h compared with **Mpi**\(^{+/+}\) controls. In cell lysates, Man-6-P inhibited hexokinase (70%), phosphoglucose isomerase (85%), and glucose-6-phosphate dehydrogenase (85%), but not phosphofructokinase. Incubating intact **Mpi**\(^{-/-}\) fibroblasts with 2-[\(^{3}H\)]deoxyglucose confirmed mannose-dependent hexokinase inhibition. Our results in vitro suggest that mannose toxicity in **Mpi**\(^{-/-}\) embryos is caused by Man-6-P accumulation, which inhibits glucose metabolism and depletes intracellular ATP. This was confirmed in E10.5 **Mpi**\(^{-/-}\) embryos where **Mpi**\(^{-/-}\) increased more than 10 times, and ATP decreased by 50% compared with **Mpi**\(^{+/+}\) littermates. Because **Mpi** ablation is embryonic lethal, a murine CDG-Ib model will require hypomorphic **Mpi** alleles.

Mannose (Man) is a major component of many glycans. Man activation first requires conversion to mannose 6-phosphate (Man-6-P), which occurs by two routes: direct phosphorylation of Man by hexokinase (HK)\(^{6}\) or interconversion from fructose 6-phosphate (Fru-6-P) via phosphomannomannase isomerase (PMI) (EC 5.3.1.8), the latter linking glycolysis to protein glycosylation. Man-6-P is converted to Man-1-P and then to GDP-mannose (GDP-Man), the central activated Man donor in glycosylation reactions (Fig. 1). PMI is encoded by the mannose-6-phosphate isomerase (PMI) gene, which has been cloned from several sources, including *Saccharomyces cerevisiae*, *Candida albicans*, mouse and human (1–4). The mouse **Mpi** gene has 8 exons covering 7.2 kb with intron-exon boundaries essentially the same as its human ortholog, with 85% amino acid identity (3). Loss of pmii40, the yeast homolog of **Mpi**, is lethal unless Man is provided in the growth medium (5). In humans, mutations in **Mpi** cause congenital disorder of glycosylation type Ib (CDG-Ib, OMIM 602579) and result in inefficient glycosylation caused by a limited amount of precursors (6). However, the clinical symptoms and aberrant glycosylation can be corrected with dietary Man supplements (6–9).

Although Man is beneficial for CDG-Ib patients, it is toxic to honeybees and becomes teratogenic to mid-stage rat embryos when given in high concentrations (10–14). The toxicity appears to stem from an accumulation of Man-6-P which cannot efficiently enter glycolysis, instead becoming trapped in a cycle of dephosphorylation and rephosphorylation resulting in depletion of intracellular ATP. This “honeybee effect” occurs in cells with HK:PMI activity ratios greater than 7, where most cells typically have ratios of 2–3 (11, 15). The physiological plasma concentration of Man in mammals, including humans and mice, ranges from 55 to 100 μM (16), but its rapid intracellular metabolism through PMI prevents Man-6-P accumulation and toxicity.

CDG-Ib patients have 3–20% residual PMI activity and suffer from hypoglycemia, coagulopathy, protein-losing enteropathy, and liver fibrosis (17, 18). All except hepatic fibrosis resolve when patients are given dietary Man supplements (6, 19). Previous experiments in rats and mice show that Man provided in the drinking water raises levels in blood and milk and is taken up by all tissues without toxic effects (16, 20, 21). Encouraged by these results, we sought to generate a mouse model of CDG-Ib in which the predicted pathology would respond to nontoxic Man therapy.

**MATERIALS AND METHODS**

*Mpi Targeting and Generation of Mice*—Embryonic stem cells from a 129/SvEv background bearing a retroviral gene trap vector (VICTR48)

6 The abbreviations used are: HK, hexokinase; 2AB, 2-aminobenzamide; AEC, aminoethylcarbazole; CDG-Ib, congenital disorder of glycosylation type Ib; ConA, concanavalin A; 2-[\(^{3}H\)]DG, 2-[\(^{3}H\)]deoxyglucose; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high pressure liquid chromatography; IUGR, intrauterine growth retardation; MEF, murine embryonic fibroblast; PGI, phosphoglucone isomerase; PMI, phosphomannomannose isomerase; RACE, rapid amplification of cDNA ends.
from each sample, and the remaining carcass was minced in 0.25% trypsin/EDTA solution (Invitrogen) with a sterile razor blade and incubated at room temperature for 5 min. Trypsin was inactivated with an equal volume of complete medium (low glucose Dulbecco’s modified Eagle’s medium (Invitrogen), 20% fetal bovine serum (Hyclone), 2 mM glutamine (Invitrogen), and 100 μg/ml penicillin/streptomycin (Invitrogen)). Cells were plated in either 60-mm (Mpi$^{+/+}$ and Mpi$^{+/−}$) or 35-mm (Mpi$^{−−}$) plates in complete medium with 100 μM Man added. Subsequent maintenance of spontaneously immortalized lines was in complete medium containing 20 μM Man.

**PMI Enzymatic Activity Assay**—PMI enzymatic assays were performed as described previously (6) except that the assay was run at 37 °C. Results are expressed as relative activities compared with Mpi$^{+/+}$. In addition, PMI activity was measured by conversion of [2-3H]Man-6-P to 3HOH (6). Briefly, 15 μg of protein from MEFs was incubated with ~200,000 cpm of [2-3H]Man-6-P in 50 mM Hepes buffer (pH 7.1) for 30 and 60 min producing 3HOH, which was evaporated in the SpeedVac. The difference in cpm between evaporated and nonevaporated samples determines the 3HOH content and is proportional to PMI activity.

**Reverse Transcription-PCR and Western Blot Analysis of PMI**—Total RNA was isolated from either whole embryos or MEFs using TRIzol reagent (Invitrogen) as per the manufacturer’s protocol. For reverse transcription-PCR, serial dilutions of total RNA were used, with 10 times the amount of RNA in null reactions. Reverse transcription was run for 30 min at 50 °C, followed by amplification of 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 60 °C for 30 s, 70 °C for 45 s, and 72 °C for 10 min. Primers were OVW252 (see above) and Mpi-5exo1-5′-GGCAACTGGGATGGCGAGTC-3′ and Mpi-5exo1-3′-AAAGCGGCCATTTTCCACCAT-3′. For loading control, primers for glyceraldehyde phosphate dehydrogenase were used: GAPDH-FOR, 5′-GGTTGCTACCTGCCCATTC-3′ and GAPDH-REV, 5′-AGGTGAGGGAAGTGGGTGTCG-3′.

**Immunohistochemistry**—Paraformaldehyde-fixed tissue sections were stained with either hematoxylin and eosin for structural analysis or with a polyclonal PMI antibody (0.5 μg/ml) for immunolocalization (3). For concanavalin A (ConA) staining, deparaffinized and peroxidase blocked sections were incubated with 100 mM methyl mannoside served as negative control. Afterward, tissue sections were incubated with horseradish peroxidase-conjugated streptavidin and developed with AEC (Zymed Laboratories Inc.).

**Production of Primary Marine Embryonic Fibroblasts (MEFs)**—MEF cultures were prepared from individual E11.5 Mpi$^{+/+}$, Mpi$^{+/−}$, and Mpi$^{−−}$ embryos. The head, limbs, and internal tissues were removed and inserted into the Mpi locus (Omnibank OST90588) were obtained from Lexicon Genetics (The Woodlands, TX). Incorporation of the VICTR48 vector, based on the VICTR20 (22), into actively transcribing genes splices 5′-exonic sequences to a neomycin resistance cassette (Fig. 2A) while simultaneously preventing splicing of the normal gene product. Retroviral insertion site was identified by sequence analysis (data not shown) to lie in intron 1 of Mpi. Neomycin-resistant embryonic stem cell clones were selected with G418 and injected into C57BL/6 blastocysts following standard protocols (23). Chimeric females (F0) were bred to C57BL/6 males to produce F1 offspring, with subsequent heterozygous interbreedings to produce experimental mice. All mice were kept on a 12-h light/12-h dark cycle.

**PCR Genotyping of Embryos and Adult Mice**—Genomic DNA from adult tail clips was isolated using a commercially available kit (Promega) following the suggested protocol. For embryo analysis, DNA was collected from lysed embryonic or yolk sac tissues. Multiplex PCR was performed for routine genotyping with the following primers and conditions (Fig. 2): Mpi primer set: P1 (oVW252), 5′-GGCAACTGGGATGGCGAGTC-3′, and P2 (CDmpi-R1), 5′-CCACCTTCCCCAGGCATC-3′; neo primer set: P3 (CDneo-F1), 5′-GATCCGGCTACCTGCCCATTC-3′, and P4 (CDneo-R1), 5′-AAAGCGGCCATTTTCCACCAT-3′. Conditions for amplification were 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. Occasionally, long range PCR with only the Mpi primer set was performed (Fig. 2B, bottom) to confirm genotype, using the following cycle conditions: 94 °C for 2 min, 30 cycles of 94 °C for 20 s, 60 °C for 30 s, 68 °C for 4 min + 20 s/cycle increase after the first 10 cycles and 68 °C for 7 min.
Ablation of Mouse Phosphomannose Isomerase

Measurement of Man-6-P and ATP in Embryos and MEFs—25 μg of embryo or MEF lysate were desalted over a carboxylate column as described earlier (24). The dried eluates were incubated with 0.35 M 2-amino-2-benzamidine (2AB, Sigma) dissolved in 30% acetic acid in dimethyl sulfoxide containing 1.0 M NaCNBH4 at 65 °C for 2 h. Free 2AB and 2AB-labeled sugars were separated over a silica gel column. 2AB-labeled sugars were separated by HPLC using a gradient with 500 mM ammonium formate, pH 4.0 (buffer) and water as the mobile phase. For the first 12 min, the buffer concentration was increased from 40 to 60%, then immediately increased to 100%, held for 10 min, and immediately returned to 40% to reequilibrate the column for another 8 min. 2AB-labeled sugars were detected at 254 nm (ext)/330 nm (em). Man-6-P eluted at 20.5 min, Glc-6-P eluted at 23 min. Man-6-P amounts in the samples were calculated according to the recovery of internal standards. Man-6-P amounts normalized to protein content in fibroblasts were adjusted to cell volumes to calculate intracellular Man-6-P concentrations. Cell volumes in Mpi−/− and Mpi+/− fibroblasts were determined using a method based on exclusion of [3H]inulin but not [14C]laurin (25).

The ATP content in embryos and fibroblasts was determined using a modified method according to Stocchi et al. (26). Briefly, fibroblasts or embryos were lysed and deproteinized with 0.5 M KOH on ice for 3 min. The pH of the lysate was adjusted to 6.5 by addition of a 1 M KH2PO4 solution. Analysis was performed on a 5-μm C18 column (25 cm × 4.6 mm inner diameter). The mobile phase used for separation consisted of two eluants: 0.1 M KH2PO4, pH 6.0, containing 8 mM tetraethylammonium hydrogen sulfate (buffer A) and buffer A containing 30% (v/v) methanol (buffer B) with a flow rate of 1.5 ml/min. The chromatographic conditions were 4 min in 100% buffer A, 4 min 0–20% buffer B, 8 min 20–40% buffer B, 6 min 40–100% buffer B and hold for 8 min. The gradient was then immediately returned to 100% buffer A and the column reequilibrated for 7 min. Nucleotides and nucleosides were detected at 254 nm. ATP eluted at 23 min.

HK, Phosphoglucoisomerase (PGI), Glc-6-P Dehydrogenase, and 6-Phosphofructo-1-kinase Activities in Cell Lysates—The effect of Man-6-P on the activities of HK, PGI, and Glc-6-P dehydrogenase were determined using an enzymatic method based on production of NADPH, measured as increasing absorbance at 340 nm. HK, PGI, and Glc-6-P dehydrogenase activities were measured by incubating cell lysates with Glc, Fru-6-P, and Glc-6-P, respectively, with saturating amounts of PGI and Glc-6-P dehydrogenase for the HK assay and Glc-6-P dehydrogenase for the PGI assay. No other enzymatic additions were needed for the Glc-6-P dehydrogenase assay. For 6-phosphofructo-1-kinase activities, a method based on the coupled enzymatic conversions of Fru-6-P to glycerol-3-P with the subsequent oxidation of NADH to NAD+ and decreased absorbance at 340 nm was used (27).

HK Activity in MEFs—The effect of Man supplementation on HK activity in MEFs was determined by measuring intracellular phosphorylation of [3H]-labeled 2-deoxyglucose (2-[^3H]DG). Briefly, after a 6-h incubation with either Man or Glc, 35 μCi of 2-[^3H]DG was added to the MEFs. Cells were trypsinized and cell lysates were applied to QAE columns. Unphosphorylated 2-[^3H]DG was eluted with 2 mM Tris buffer. Phosphorylated 2-[^3H]DG was eluted with 2 mM Tris containing 125 mM NaCl.
RESULTS

Ablation of Mpi—PMI deficiency in humans causes CDG-Ib, and the clinical symptoms can be mostly corrected with Man supplementations (6). To study the effects of PMI deficiency and Man treatment, we aimed at generating a mouse model of CDG-Ib. We obtained a commercial line of mice harboring a disruption of Mpi (Fig. 2A). The VICTR48 retroviral insertion prevents normal splicing of exons 1 and 2, instead producing an exon1-neo fusion protein, and is predicted to eliminate completely the normal Mpi gene product. Mpi<sup>+/+</sup>, Mpi<sup>+/−</sup>, and Mpi<sup>−/−</sup> offspring were identified by multiplex PCR, with occasional long range PCR to confirm genotypes (Fig. 2B). To determine the efficiency of gene disruption, we collected mid-stage embryos (E11.5) from heterozygous matings and analyzed them for PMI expression and enzyme activity (Fig. 3). Both PMI mRNA (Fig. 3, A and B) and protein levels (Fig. 3C) were reduced by ~50% in Mpi<sup>−/−</sup> compared with Mpi<sup>+/+</sup>. Neither PMI mRNA nor protein expression could be detected in Mpi<sup>−/−</sup> embryos (<0.1% of Mpi<sup>+/+</sup>). Although measurement of PMI enzymatic activities using a traditional in vitro coupled assay (6) revealed that Mpi<sup>−/−</sup> samples had ~5% of Mpi<sup>+/+</sup> activity, a highly sensitive PMI assay that measures the conversion of [2-<sup>3</sup>H]-Man-6-P to 3HOH (6) showed that the activity in Mpi<sup>−/−</sup> samples was less than 0.01% compared with Mpi<sup>+/+</sup> samples. Mpi<sup>+/+</sup> samples had 49.1% remaining activity (Fig. 3D).

Phenotypic Effects of Mpi Ablation—Animals heterozygous for Mpi ablation were viable and fertile and generally appeared normal compared with wild type littermates. Serum chemistry (blood glucose, blood urea nitrogen, creatinine, bicarbonate, chloride, sodium, potassium, calcium, total protein) and hematological (white blood count, red blood count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, mean platelet volume, platelet count) parameters were normal. Mice used for intercrosses were maintained on normal water and chow or were placed on 10% Man for 2 weeks prior to mating. Consistent with previous studies in wild type mice (16), Man treatment did not affect the average litter size or male:female ratios of pups that survived to weaning (Table 1). Additionally, normal Mendelian ratios between Mpi<sup>+/+</sup> and Mpi<sup>+/−</sup> pups were observed. In contrast, no Mpi<sup>−/−</sup> mice were recovered of 380 pups without Man treatment, or 126 mice born to females maintained on 10% Man supplements during gestation (Table 1). These data indicate a prenatal loss of Mpi<sup>−/−</sup> embryos which does not respond to Man supplementation.

We examined prenatal litters of various stages to determine when embryonic lethality commenced. Normal Mendelian ratios were observed up to E9.5 with very few resorptions (Fig. 4A and Table 1). However, resorptions began to increase at E11.5 with a corresponding decrease in the number of recoverable Mpi<sup>−/−</sup> embryos. Analysis at E13.5 revealed a total absence of Mpi<sup>−/−</sup> embryos with a further increase in resorptions (31%), which was consistent with the lack of Mpi<sup>−/−</sup> pups at term.

Embryonic lethality caused by Mpi ablation prompted us to provide Man to the dams, anticipating prolonged survival of Mpi<sup>−/−</sup> embryos. Contrary to expectations, providing females with water containing 10% Man before mating and during development significantly increased the number of resorptions and eliminated Mpi<sup>−/−</sup> embryos from the E11.5 population (Fig. 4, B and C). Initiating 3% (<i>n</i> = 54) or 10% Man (<i>n</i> = 41) supplements at E9.5 to rescue the embryos did not reduce the number of resorptions at E11.5 (data not shown). These results suggest that early Man supplementation accelerates embryonic lethality in Mpi<sup>−/−</sup> mice.

**TABLE 1**

Genotypic distribution and litter sizes from heterozygous matings

| Sample     | Genotype | Mean litter size<sup>a</sup> (N) | Male:female ratio |
|------------|----------|----------------------------------|------------------|
| 3-week-old pups |          |                                  |                  |
| No mannose | +/+      | 130                              | 0.8              |
|            | +/−      | 250                              | 0.8              |
|            | −/−      | 0                                | 0.8              |
| 10% mannose | +/+      | 44                               | 0.8              |
|            | +/−      | 82                               | 0.8              |
|            | −/−      | 0                                | 0.8              |
| Embryo stage (dpc)<sup>b</sup> |          |                                  |                  |
| 8.5        | +/+      | 8                                | 1.5              |
|            | +/−      | 8                                | 1.5              |
|            | −/−      | 6                                | 1.5              |
| 9.5        | +/+      | 4                                | 1.5              |
|            | +/−      | 10                               | 1.5              |
|            | −/−      | 6                                | 1.5              |
| 11.5       | +/+      | 22                               | 1.2              |
|            | +/−      | 44                               | 1.2              |
|            | −/−      | 17                               | 1.2              |
| 13.5       | +/+      | 5                                | 1.0              |
|            | +/−      | 11                               | 1.0              |
|            | −/−      | 0                                | 1.0              |

<sup>a</sup> For embryos, includes resorption sites and litters not genotyped.

<sup>b</sup> Days postcoitus.
Ablation of Mouse Phosphomannose Isomerase

FIGURE 4. Ablation of Mpi produces embryonic lethality by mid-gestation that is accelerated with mannose treatment. A, litters harvested from Mpi+/− intercrosses at various mid-gestational stages of development were scored for the number of resorptions (triangles) and number of Mpi−/− embryos (squares) present. B and C, further analysis of E11.5 litters in response to supplying mares with 10% Man in the drinking water was performed and scored similarly for the number of resorptions (B) and number of Mpi−/− embryos (C) present. Man treatment was commenced 2 weeks prior to mating and maintained throughout gestation. Dark and light gray bars represent untreated and 10% Man treated, respectively. Data points (A) and bars (B and C) are labeled as the number of occurrences of total number examined from each group and depicted as percent of total.

Gross inspection of Mpi−/− embryos revealed normal development up to E8.5. However, at E9.5 intrauterine growth retardation (IUGR) occurred, represented by two general phenotypes. One set of Mpi−/− embryos (group 1) appeared similar to the E9.0 stage of development (15–18 pairs of somites) and resembled untreated E8.5 embryos (data not shown). The IUGR continued to E11.5 where the first group of Mpi−/− embryos was developmentally similar to E10.0–10.5 (30–35 pairs of somites), whereas the second group, 35% of all E11.5 Mpi−/− embryos, resembled E8.5–9.0. At this stage some of the embryos from this second group did complete axial turning, a developmental milestone. However, all of these group 2 Mpi−/− embryos displayed posterior axial truncation and apparent failure of chorioallantoic fusion. Yolk sac vascular branching was abnormal in more than 90% of E11.5 Mpi−/− embryos, with complete absence in all of the group 2 Mpi−/− embryos.

The IUGR and chorioallantoic/yolk sac abnormalities observed in Mpi−/− embryos are similar to those that develop from insufficient placentaion (28). We therefore examined placentas from Mpi−/− embryos that completed chorioallantoic fusion (group 1). E11.5 Mpi−/− placentas showed decreased lateral extension with increased placental bed thickness compared with control littersmates (Fig. 5, J and K), with a substantial decrease in lateral extension. Histological analysis revealed hypertrophic expansion of the trophoblast giant cell, spongiotrophoblast, and, to a lesser extent, labyrinthine layers (Fig. 5M). Embryonic vascular penetration into the labyrinthine layer (as evidenced by vessels containing immature, nucleated red blood cells) was comparable to control littersmates (Fig. 5L), but the overall architecture of the placental layers was more disorganized (Fig. 5M). ConA staining was identical in Mpi−/− and control placentas (data not shown).

Defective embryonic vascular patterns are also often associated with IUGR and problems with placentaion (29). Analysis of embryonic vasculature in Mpi−/− embryos utilizing whole mount CD31 immunohistochemistry revealed cephalic vasculature abnormalities defined by premature termination of the vessels (Supplemental Fig. S1B, arrow), which likely produces the cephalic hemorrhage sometimes seen in group 1 embryos (Fig. 5E). However, despite the cranial vascular defects, overall vascular patterning appeared normal (Supplemental Fig. S1D). E9.5 group 1 embryos displayed normal development of the cranial primary vascular plexus (data not shown), suggesting that the problems noted at E11.5 resulted from defective angiogenesis and not a vasculogenic problem. In contrast, Mpi−/− group 2 embryos displayed extensive vascular deficiencies, with fragmented vessels throughout the embryos (Supplemental Fig. S1E).

Histological and immunohistochemical comparisons between Mpi−/− embryos and control littersmates at E10.5 suggested that organogenesis proceeded correctly in group 1 embryos (Fig. 6B), but...
the stage of organ development was retarded. However, the organogenic delay was consistent with the IUGR. PMI antigen is normally expressed ubiquitously in embryos at this stage (Fig. 6, inset), with increased levels in the heart (Fig. 6C). As expected, PMI was not detected in Mpi−/− embryos (Fig. 6D). However, loss of PMI had no effect on N-glycosylation because ConA binding was comparable with that of control littermates (Fig. 6, E and F). Apoptosis was minimal in group 1 embryos at this stage compared with control, but it was slightly higher in the neural mesenchyme and liver primordium at E11.5 (data not shown). In contrast, extensive apoptotic foci developed throughout group 2 Mpi−/− embryos at E11.5 (Fig. 6I). Similar to group 1 embryos, ConA staining is normal in these embryos, demonstrating that death of Mpi−/− embryos is independent of a glycosylation insufficiency.

FIGURE 5. Loss of PMI produces IUGR and placental abnormalities. Gross morphology of Mpi+/+, Mpi+/−, and Mpi−/− embryos at E9.5 (A–C) and Mpi+/+, Mpi+/−, and Mpi−/− embryos at E11.5 with yolk sacs removed (D–F), and intact (G–I). Cephalic hemorrhage (arrowhead) and pericardial effusion (arrow) are indicated in E and F. Gross morphology (J and K) and H&E-stained sections (L and M) of placentas from control (J and L) and Mpi−/− (K and M) embryos are shown at identical magnifications. The outline of Mpi−/− placenta in K is superimposed on control placenta in J to show the overall reduction in size. Labyrinth layer (LaTr), spongiotrophoblast layer (SpTr), and trophoblast giant cell layer (GCTr) are identified in L and M, demonstrating the hypertrophy and disorganized architecture of mutant placentas. The region of chorioallantoic connection is at the top of the panel in L and M. Scale bar = 1 mm (A–K) and 400 μm as noted (L and M).
Biochemical Analysis of Mpi<sup>−/−</sup> Embryos—One explanation for the loss of Mpi<sup>−/−</sup> embryos is that physiological levels of Man are toxic and that further raising Man concentrations in the mother’s blood increases embryonic exposure to Man, promoting earlier toxicity. Normal physiological levels of blood mannose in mice are ~100 μM, and 10% Man supplements raise it to nearly 500 μM (16). Although we could not obtain Mpi<sup>−/−</sup> embryos from Man-supplemented mice, analysis of E10.5 embryos from females given plain water revealed that Mpi<sup>−/−</sup> embryos had a 10-fold increase of Man-6-P, whereas neither Mpi<sup>+/+</sup> (data not shown) nor Mpi<sup>++/−</sup> embryos had levels above background (Fig. 7A).

Because previous studies describing the honeybee effect show a futile cycle of phosphorylation and dephosphorylation of Man and Man-6-P which depletes ATP, we also measured ATP concentrations in E10.5 embryos. We did not observe any differences between Mpi<sup>+/+</sup> and Mpi<sup>++/−</sup> embryos, but ATP was 50% lower in Mpi<sup>−/−</sup> embryos compared with Mpi<sup>+/+</sup> (p < 0.01) (Fig. 7B). This supports the hypothesis that accumulation of Man-6-P causes death of Mpi<sup>−/−</sup> embryos through ATP depletion.

Effects of Man on Mpi<sup>−/−</sup> MEFs—To investigate the basis of mannose toxicity in Mpi<sup>−/−</sup> embryos, we determined the related effects of Man on growth and survival of MEFs isolated from Mpi<sup>+/+</sup> and Mpi<sup>++/−</sup> embryos. Because normal cell growth requires Man for glycoprotein synthesis, and “excess” Man is probably toxic to Mpi<sup>−/−</sup> cells, it was important to optimize the amount of Man available to the cells. In addition to direct transporter-mediated uptake of extracellular Man, it can be derived from intracellular catabolism or processing of oligosaccharides or Man-containing endocytosed serum glycoproteins. We therefore compared the growth of Mpi<sup>++/−</sup> and Mpi<sup>−/−</sup> cells in the presence of different concentrations of fetal bovine serum (FBS) and

FIGURE 6. Loss of PMI does not affect embryonic glycan production. Sagittal sections of E10.5 Mpi<sup>++/−</sup> (A, C, and E) and Mpi<sup>++/−</sup> (B, D, and F) embryos are stained with H&E (A and B), anti-PMI (C and D), and ConA (E and F) as described under “Materials and Methods.” C–F are high magnification and only show hearts. Whole embryo anti-PMI staining is shown in C, inset, with the boxed region within inset outlining the heart shown in C. Adjacent sagittal sections of a group 2 E11.5 Mpi<sup>++/−</sup> embryo are stained with H&E (G), anti-PMI (H), and Tunel (I), demonstrating that the severe apoptosis that develops is independent of the normal glycosylation status. Scale bar = 1 mm.
Ablation of Mouse Phosphomannose Isomerase

Man. Mpi+/+ cells, in 20% or 100% FBS, were indifferent to exogenous Man (Fig. 8E). In contrast, Mpi−/− cells remained viable, but were unable to grow in 20% FBS without exogenous Man (Fig. 8A). Addition of 20 μM Man to the medium produced a growth rate nearly equivalent to Mpi+/+ cells. Mpi−/− cells grew slightly slower at the physiological concentration of 100 μM Man, but much slower in 500 μM Man, suggesting toxicity. Increasing FBS to 100% allowed Mpi−/− cells to grow very well without any Man supplements and slightly better with additional 20 μM Man, but growth was again compromised with 500 μM Man (Fig. 8B) compared with Mpi+/+ cells (Fig. 8G). This demonstrates that FBS can substitute for Man needed for cell growth, presumably for glycoproteins.

To verify our hypothesis that available Man resulted from catabolism of Man-containing oligosaccharides, we added the α-mannosidase inhibitors swainsonine (20 μg/ml), kifunensine (50 μg/ml), and deoxy-mannojirimycin (20 μg/ml) to the tissue culture medium. The inhibitors had little effect on Mpi+/+ cells (Fig. 8, F and H), but they prevented growth of Mpi−/− cells incubated without Man and eventually caused death (Fig. 8, C and D). Growth of Mpi−/− cells was rescued with 50 μM Man, but higher concentrations inhibited growth, again suggesting Man toxicity.

 Fate of Man in Mpi−/− MEFs—To determine the fate of exogenous Man in Mpi+/+ and Mpi−/− cells, we incubated fibroblasts with [2-3H]Man and analyzed the products. Most of the labeled [2-3H]Man entering Mpi+/+ cells (>95%) was catabolized to Fru-6-P through PMI releasing 3HOH, which was recovered in the medium. About 95% of cell-associated label was incorporated into trichloroacetic acid-precipitable material, with less than 1% remaining as various metabolic precursors. Less than 0.1% of label was found in [2-3H]Man-6-P. In contrast, Mpi−/− cells produced no 3HOH but accumulated 85–90% of the label as [2-3H]Man-6-P, 1% was free [2-3H]Man, and ~0.5% was [2-3H]Man-1-P. The remaining label was incorporated into macromolecular material. There was no increase of labeled 2-keto-3-deoxy-d-glycero-d-galactononic acid, which can be produced from Man-6-P and phosphoenolpyruvate. Pulse-chase labeling experiments show that [2-3H]Man-6-P accumulated within 30 min and was then slowly converted to [2-3H]Man (t1/2 = ~3 h), which mostly appeared in the medium.

Quantitation of Man-6-P in Mpi−/− cells revealed a concentration-dependent accumulation that reached 10 nmol/100 μg of protein at 500 μM Man (Fig. 9A). Based on cell volume measurements (270 ± 24 μg of protein/μl), this was equivalent to an intracellular Man-6-P concentration of 28 mM at 500 μM and more than 18 mM at 100 μM, the physiological level of Man. The volume of Mpi+/+ cells (247 ± 14 μg of protein/μl) was not significantly different from Mpi−/− cells. In addition, a time-dependent accumulation of Man-6-P was observed in Mpi−/− cells incubated with 500 μM Man, attaining steady-state levels after 8 h (Fig. 9B). In Mpi+/+ cells, Man-6-P concentrations did not increase after Man treatment and remained near base-line level.

Effects of Man-6-P on Mpi−/− MEFs—Consistent with energy depletion via the honeybee effect and similar to our observations in embryos, ATP was reduced in Mpi−/− cells after exposure to 500 μM Man. Although the amount of ATP was relatively stable for the first 4 h, a decrease occurred after an 8-h incubation, reaching ~25% initial levels after 24 h (Fig. 9C). Notably, ATP depletion began when maximum levels of Man-6-P had been reached.

The addition of 500 μM Man to Mpi−/− cells had no effects on DNA or protein synthesis, as demonstrated by [3H]thymidine and [35S]methionine incorporation (data not shown). However, a slight decrease in [35S]methionine incorporation was observed after 24 h of Man treatment. Within the first 24 h, exposing Mpi−/− cells to 500 μM Man did not increase the number of apoptotic cells compared with Mpi+/+ MEFs (data not shown).

To understand the mechanisms that link intracellular Man-6-P accumulation and ATP depletion, we investigated the ability of Man-6-P to perturb glycolytic flux and measured whether high Man-6-P concentrations affect various glycolytic enzymes in cell lysates from Mpi+/+ MEFs (Fig. 10A). Increasing Man-6-P inhibited HK and PGI but not phosphofructokinase. 28 mM Man-6-P, the concentration measured in MEFs after incubation with 500 μM Man, inhibited HK activity by ~75% and PGI activity by ~60%. Furthermore, Glc-6-P dehydrogenase, the enzyme catalyzing the initial step in the pentose phosphate pathway, was inhibited by more than 80% in the presence of 28 mM Man-6-P.

To verify that the addition of Man inhibits HK activity in intact fibroblasts, we determined intracellular HK-dependent phosphorylation of 2-deoxyglucose. After a 6-h preincubation with either 500 μM Man or 500 μM Glc as control, we added 2-[3H]DG to the tissue culture medium and measured intracellular 2-[3H]DG phosphorylation over time because it is not catabolized further. Because unphosphorylated 2-[3H]DG rapidly exchanges between intra- and extracellular space and phosphorylated 2-[3H]DG becomes trapped inside the cell (data not shown), total intracellular [3H] is an indirect measurement for 2-[3H]DG phosphorylation. Although preincubation with 500 μM Glc had no effect on 2-[3H]DG accumulation in Mpi+/+ cells, 500 μM Man decreased 2-[3H]DG accumulation and therefore HK activity by 70% (Fig. 10B). This effect was similar to HK inhibition in cell lysates in the presence of 28 mM Man-6-P (Fig. 10A). As seen in Fig. 10C, even physiological levels of 100 μM Man reduced 2-[3H]DG accumulation by
FIGURE 8. Effect of Man on growth of Mpi<sup>+/+</sup> and Mpi<sup>-/-</sup> MEFs. MEFs isolated from Mpi<sup>-/-</sup> (A–D) and Mpi<sup>+/+</sup> (E–H) E11.5 embryos were plated in 96-well plates and incubated with either 20% (A, C, E, and F) or 100% (B, D, G, and H) FBS, and increasing concentrations of Man without (A, B, E, and G) or with (C, D, F, and H) the addition of α-mannosidase inhibitors. Cell growth was assessed by crystal violet staining (refer to "Materials and Methods"). Man concentrations used are represented as follows: diamond, 0 μM; circle, 20 μM; square, 50 μM; ×, 100 μM, and triangle, 500 μM. Samples treated with α-mannosidase inhibitors are depicted similarly except with open symbols and lines as follows: dashed line, 0 μM; dotted line, 50 μM, and hatched line, 500 μM.
Ablation of Mouse Phosphomannose Isomerase

FIGURE 9. Man-6-P accumulates in Mpi<sup>−/−</sup> MEFs while ATP decreases. A, Mpi<sup>−/−</sup> MEFs were incubated with different concentrations of Man for 24 h. Man-6-P from MEF lysates was measured by a simpler PMI-specific assay based on the conversion of [2-<sup>3</sup>H]Man-6-P to Fru-6-P and <sup>3</sup>HOH (Fig. 3). B, Man-6-P concentrations were given as nmol/100 μg protein and are given as percent ATP in MEF lysates. C, Man-6-P concentrations were given as percent ATP in MEF lysates. 28 mM Man-6-P (dashed line), the concentration of Man-6-P predicted for cells incubated with 500 μM Man, inhibited HK activity by ~75%, phosphoglucone isomerase activity by ~60%, and Glc-6-P dehydrogenase activity by ~80%. Man supplementation decreases HK activity in intact MEFs.

Together, these results suggest that excessive Man-6-P may alter several important metabolic pathways required to maintain energy and reducing potential homeostasis in vivo and may contribute to the lethal phenotype in Mpi<sup>−/−</sup> embryos.

DISCUSSION

PMI is encoded by a single gene on chromosome 9 (32.0 cM). Although two enzymatic isoforms have been identified based on electrophoretic mobility (30), no known orthologs exist in mice, indicating that the isoforms are polymorphic variations between mouse strains. Disruption of the Mpi locus should therefore completely ablate PMI enzymatic activity. In using a gene trap strategy to ablate gene function, the trapping vector may be skipped during splicing and produce some normal product with complete activity (31). Although we measured ~5% residual PMI activity in Mpi<sup>−/−</sup> embryos and MEFs using a traditional coupled assay, Mpi<sup>−/−</sup> MEFs produced <0.01% normal activity when measured by a simpler PMI-specific assay based on the conversion of [2-<sup>3</sup>H]Man-6-P to Fru-6-P and <sup>3</sup>HOH (Fig. 3D), supporting that...
Ablation of Mouse Phosphomannose Isomerase

...the remaining activity in the coupled assay was background. The similarities in activities from the coupled assays among Mpi+/+, Mpi+/−, and Mpi−/− embryos and MEFs suggest that MEFs are representative of whole embryo activities. Furthermore, semiquantitative analyses of PMI mRNA and protein produced from Mpi−/− samples were less than 0.1% (our detection limit) compared with Mpi+/+ (Fig. 3, A–C), supporting complete ablation of the gene product at the transcriptional level. Together, these results prove that Mpi is knocked out in our mouse model with complete loss of PMI activity.

In humans, PMI mutations cause CDG-Ib because of reduced PMI activity and impaired glycosylation (17, 18). Loss of PMI prevents the de novo biosynthesis of Man-6-P from Glc, but Man in the plasma bypasses the PMI deficit and generates sufficient Man-6-P to maintain glycosylation (Fig. 1). In contrast to human deficiencies, glycosylation was normal in Mpi−/− embryos (Fig. 6, E, F, and H). Despite normal glycosylation, Mpi−/− embryos died around E11.5 (Fig. 4A). Additionally, MEF supplementation, which effectively treats CDG-Ib patients, accelerated the death of Mpi−/− embryos (Fig. 4, B and C). Together, these results suggest that Man toxicity causes the Mpi−/− lethal phenotype.

Analysis of Man metabolism in MEFs isolated from Mpi−/− and Mpi+/+ embryos confirmed the toxic effects of Man on cell proliferation. Mpi−/− MEFs grew nearly as well as Mpi+/+ cells when maintained in a medium containing 10% FBS and 20 μM Man (Fig. 8). However, increasing the Man concentration progressively retarded Mpi−/− cell growth, demonstrating Man toxicity. Complete removal of Man from the growth medium also inhibited cell growth, but this could be overcome by increasing the amount of serum to 100% (Fig. 8D), suggesting that cells were able to salvage Man from degraded, endocytosed glycoproteins and oligosaccharides. This source of Man reutilization was confirmed by the addition of α-mannosidase inhibitors to serum-supplemented Mpi−/− MEFs to remove all sources of Man completely, which resulted in cell death (Fig. 8, C and D). As expected, cells could be rescued by providing 50 μM Man along with the inhibitors, but further increases in exogenous Man inhibited Mpi−/− cell growth. These results indicate that, although a source of Man is essential for survival, an excess amount of Man becomes toxic in the absence of PMI. The toxic effect of Man and subsequent Man-6-P accumulation on Mpi−/− cell growth was also seen in yeast lacking the homolog of PMI (32).

Our results from MEFs can explain why Man supplementation hastens embryonic lethality. Based on previous studies defining the honeybee effect, we hypothesized that an excess of Man causes Man-6-P accumulation because it cannot be converted to Fru-6-P through PMI for further catabolism. Here we show for the first time in mice that accumulated Man-6-P depletes ATP not only through a futile cycle of Man-6-P further catabolism. Here we show for the first time in mice that accumulated Man-6-P depletes ATP not only through a futile cycle of Man-6-P, but Man in the plasma also inhibits cell growth, demonstrating Man toxicity. Complete removal of Man from the growth medium also inhibited cell growth, but this could be overcome by increasing the amount of serum to 100% (Fig. 8D), suggesting that cells were able to salvage Man from degraded, endocytosed glycoproteins and oligosaccharides. This source of Man reutilization was confirmed by the addition of α-mannosidase inhibitors to serum-supplemented Mpi−/− MEFs to remove all sources of Man completely, which resulted in cell death (Fig. 8, C and D). As expected, cells could be rescued by providing 50 μM Man along with the inhibitors, but further increases in exogenous Man inhibited Mpi−/− cell growth. These results indicate that, although a source of Man is essential for survival, an excess amount of Man becomes toxic in the absence of PMI. The toxic effect of Man and subsequent Man-6-P accumulation on Mpi−/− cell growth was also seen in yeast lacking the homolog of PMI (32).

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It is unclear why Mpi−/− embryos can survive to E11.5 if Man toxicity is the result of Man-6-P accumulation and ATP depletion. It may be caused by a cascade of developmental defects. The IUGR, yolk sac defects, and failure of chorioallantoic fusion seen in Mpi−/− embryos (Fig. 5) are typical phenotypic presentations of inadequate placentaion (29). Although it is difficult to ascribe a specific mechanism, glycolytic insufficiency may perturb proliferation of the extraembryonic mesoderm needed for chorioallantoic fusion and choriovitelline (yolk sac) placentaion (28). Mice lacking glucoseposphate isomerase (Gpi), (33) have impaired glycolsis and develop severe mesodermal defects, whereas both endoderm and ectoderm are relatively normal. In fetal chimeras, Gpi-deficient cells populate the yolk sac endoderm layer more efficiently compared with the yolk sac mesoderm layer (34), suggesting that extraembryonic mesoderm is more sensitive to glycolytic disturbances than the endoderm. Yolk sac vascular patterning also requires extraembryonic mesoderm for proper development and maintenance (35). Together, this suggests that both group 1 (yolk sac defect) and group 2 (chorioallantoic fusion block) Mpi−/− embryos can result from problems with the extraembryonic mesoderm, which generates both yolk sac and chorioallantois.

Group 1 Mpi−/− embryos show placental hypertrophy at E11.5. One possible explanation may be that the spongiosplastblastuloblast proliferates in response to the increased hypoxic environment that likely develops in Mpi−/− embryos because of yolk sac failure. Hypoxia promotes the differentiation of trophoblast stem cells into spongiosplastblastuloblast fates (36). Increased proliferation of the spongiosplastblastuloblast can be forced into the labyrinthine zone, producing the less organized and hyper- trophic appearance seen in Mpi−/− placentas.

The two observed embryonic phenotypes could also be attributed to strain effects because the experiments were performed on mice with a mixed background (129, C57BL/6).

Impaired PMI activity as seen in CDG-Ib patients cannot be mimicked in the mouse by ablation of Mpi and complete loss of PMI activity. Although Man-6-P is the limiting glycosylation precursor in CDG-Ib patients, it becomes toxic in the mouse when PMI is lost completely. A minimum of PMI activity seems necessary to “detoxify” the cells from accumulating Man-6-P. CDG-Ib fibroblasts growing in media containing up to 500 μM Man do not accumulate Man-6-P. However, PMI expression is variable (3), and the HK:PMI ratio will influence Man-6-P levels. For instance, PMI expression is extremely high in round spermatids that have low HK activity and glycolytic flux, but in spermatozoa, the ratio is reversed. Depending on a patient’s residual PMI activity, Man supplements could conceivably alter spermatogenesis. Although no adult CDG-Ib patients are currently receiving supplements, this issue should be kept in mind and warrants establishing a CDG-Ib mouse model. Because Mpi ablation is embryonic lethal, a murine CDG-Ib model will require hypomorphic Mpi alleles.

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Ablation of Mouse Phosphomannose Isomerase

MARCH 3, 2006 • VOLUME 281 • NUMBER 9

JOURNAL OF BIOLOGICAL CHEMISTRY 5927

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