Respiratory Distress and Neonatal Lethality in Mice Lacking Golgi α1,2-Mannosidase IB Involved in N-Glycan Maturation*

Received for publication, September 7, 2006, and in revised form, October 31, 2006. Published, JBC Papers in Press, November 22, 2006, DOI 10.1074/jbc.M608661200

Linda O. Tremblay†, Erzsebet Nagy Kovács†, Eugene Daniels‡, Nyet Kui Wong*, Mark Sutton-Smith*, Howard R. Morris*, Anne Dell†, Edwige Marcinkiewicz†, Nabil G. Seidah†, Colin McKerlie**, and Annette Herscovics††

From the †McGill Cancer Centre and the **Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, United Kingdom, the †Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montréal, Montréal, Québec H2W 1R7, Canada, and the **Program in Lung Biology Research, The Hospital for Sick Children and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5G 1X8, Canada

There are three mammalian Golgi α1,2-mannosidases, encoded by different genes, that form Man₅GlcNAc₂ from Man₉GlcNAc₂ for the biosynthesis of hybrid and complex N-glycans. Northern blot analysis and in situ hybridization indicate that the three paralogs display distinct developmental and tissue-specific expression. The physiological role of Golgi α1,2-mannosidase IB was investigated by targeted gene ablation. The null mice have normal gross appearance at birth, but they display respiratory distress and die within a few hours. Histology of fetal lungs the day before birth indicate some delay in development, whereas neonatal lungs show extensive pulmonary hemorrhage in the alveolar region. No significant histopathological changes occur in other tissues. No remarkable ultrastructural differences are detected between wild type and null lungs. The membranes of a subset of bronchiolar epithelial cells are stained with lectins from Phaseolus vulgaris (leuкоagglutinin and erythrosagglutinin) and Datura stramonium in wild type lungs, but this staining disappears in lungs from null mice. Mass spectrometry of N-glycans from different tissues shows no significant changes in global N-glycans of null mice. Therefore, only a few glycoproteins required for normal lung function depend on α1,2-mannosidase IB for maturation. There are no apparent differences in the expression of several lung epithelial cell and endothelial cell markers between null and wild type mice. The α1,2-mannosidase IB null phenotype differs from phenotypes caused by ablation of other enzymes in N-glycan biosynthesis and from other mouse gene disruptions that affect pulmonary development and function.

* This work was supported by the Canadian Institutes of Health Research (to A. H. and N. G. S.) and by the Mizutani Foundation (to A. H.). Work at the Imperial College was supported by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: 3655 Promenade Sir William Osler, Montréal, Québec H3G 1Y6, Canada. Tel.: 514-398-3533; Fax: 514-398-6769; E-mail: annette.herscovics@mcgill.ca.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.
in Refs. 20 and 21). Furthermore, the unique cell- and tissue-specific roles of individual members of multigene enzyme families have also been revealed by these studies. For example, α-mannosidase II-deficient mice develop dyserythropoietic anemia and autoimmune disease (22, 23), yet disruption of the mouse α-mannosidase II gene results in hypoperoctogenesis and male infertility (24). However, ablation of both α-mannosidase II and IIx completely abrogates complex N-glycan biosynthesis with the accumulation of hybrid structures, and causes hepatic and pulmonary ultrastructural defects. Although a few of these mice survive (> P21), the majority die within 2 days of birth and some die in utero (25). These animal models have provided insights into critical functions mediated by N-glycans that cannot be observed in cultured cells. This was first noted by the ability to grow cells lacking N-acetylglucosaminyltransferase I in culture (26), whereas gene ablation is embryonic lethal in the mouse (2, 3).

The physiological roles of the different mammalian Golgi α1,2-mannosidases in vivo have not been previously established. In the present work we report that inactivation of Golgi α1,2-mannosidase IB generates mice with a distinct phenotype. Golgi α1,2-mannosidase IB null mice develop to term, and display no apparent gross abnormality at birth. However, shortly after birth they exhibit signs of respiratory distress and die within several hours with evident lung hemorrhage. This phenotype demonstrates the specific role of Golgi α1,2-mannosidase IB in pulmonary function and development.

**EXPERIMENTAL PROCEDURES**

Golgi α1,2-Mannosidase Transcript Expression—Northern blot analysis was performed using α1,2-mannosidase IA, IB, and IC probes amplified from mouse cDNA by PCR using the following primer sets: ManIA, 5’-CCTCCTGGAACAC-AAGATG-3’ and 5’-AAACAAATTTGAGACTTATGAGG-3’; ManIB, 5’-GATAGTTTTTATGTAATACTTACTG-3’ and 5’-GTGCGCCTCTGTGTTAAAC-3’; ManIC, 5’-GTCTTATG-CGGCTTCAGA-3’ and 5’-CTGTCTCTCCTCAGAGGAA-3’. The probes were labeled with [α-32P]dATP (3000 Ci/mmol) using the megaprime labeling kit (Amersham Biosciences). The probes were sequentially transfected with 10 μg of supercoiled Cre expression plasmid (pIC-Cre; gift of Dr. F. R. Jirik) by electroporation. Cells were initially plated at dilutions of 1/30 and 1/90. After 2–3 days of growth the cells were trypsinized, plated at dilutions of 1/100 and 1/150, and selected with 0.2 μM 2’-deoxy-2’-fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU) (Moravek Biochemicals) for 11 days. Cre excision of the loxP flanked exon and tk-neo cassette was established by Southern blot analysis of FIU-resistant clones. ES cells containing Type I (systemic) and Type II (conditional) allelic deletions were obtained.

Chimeric mice were generated by microinjection of two independent Type I ES cell clones into 3.5-day C57BL/6 blastocysts using standard techniques (29, 30). Heterozygous null allele mice were obtained by crossing the chimeric males with C57BL/6 or 129Sv females. The heterozygous mice were then intercrossed to obtain α1,2-mannosidase IB-deficient mice. Heterozygous mice on a mixed 129Sv and C57BL/6 background were also backcrossed for eight generations onto the CD-1 background.

DNA Isolation and Southern Blot Analysis—ES cells and tail clippings were lysed overnight at 37 or 55 °C, respectively, in 100 mM Tris, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 0.4 mg/ml proteinase K. The samples were then incubated with RNase A at 37 °C for 1 h, and ES cell samples were also treated with 0.4 mg/ml proteinase K at 55 °C for several hours. The DNA was either precipitated directly by the addition of an equal volume of isopropanol alcohol, or with 2 volumes of ethanol following phenol and chloroform extractions.

Genotyping of ES cells and mice was performed by hybridization of [α-32P]dATP (3000 Ci/mmol) labeled probes to

2 The abbreviations used are: FIUA, 2’-deoxy-2’-fluoro-β-D-arabinofuranosyl-5-iodouracil; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ACTH, adrenocorticotropic hormone; RT, reverse transcriptase; E, embryonic day; PHA, phytohemagglutinin; VEGF, vascular endothelial growth factor; P, postnatal day; PECAM, platelet endothelial cell adhesion molecule.

fixed in 4% formaldehyde. The sections were exposed to Kodak BioMax film for 24 h, as described previously (27, 28).

α1,2-Mannosidase IB Gene Ablation—The α1,2-mannosidase IB targeting construct was prepared by inserting genomic DNA isolated from a 129/ola P1 mouse library (Genome Systems) (14) into the pflox vector (gift of Dr. J. D. Marth). A 2.6-kb BglII fragment containing exon 2 was ligated into the BamHI site of pflox, and the flanking Stul-BglII (2.3 kb) and BglII-Spel (2.8 kb) genomic fragments were then ligated into the Sall and Xhol sites of the vector, respectively (Fig. 3A).

The targeting construct (40 μg) was linearized with NotI and introduced into J1 ES cells by electroporation (29, 30). The cells were plated and selected with G418 (300 μg/ml; Invitrogen) for 10 days. The ES cells were cultured in LIF supplemented medium on a feeder layer of γ-irradiated mouse embryonic fibroblasts in gelatinized tissue culture dishes.

Homologous recombinant clones were identified by Southern blot analysis using genomic probes located 5’ and 3’ to the targeted allele (Fig. 3B), as well as a loxP probe (31) and a neo probe derived from the pflox vector (1.2-kb NcoI fragment).

Five homologous recombinant clones (105 cells) were transiently transfected with 10 μg of supercoiled Cre expression plasmid (pIC-Cre; gift of Dr. F. R. Jirik) by electroporation. Cells were initially plated at dilutions of 1/30 and 1/90. After 2–3 days of growth the cells were trypsinized, plated at dilutions of 1/100 and 1/150, and selected with 0.2 μM 2’-deoxy-2’-fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU) (Moravek Biochemicals) for 11 days. Cre excision of the loxP flanked exon and tk-neo cassette was established by Southern blot analysis of FIU-resistant clones. ES cells containing Type I (systemic) and Type II (conditional) allelic deletions were obtained.

Chimeric mice were generated by microinjection of two independent Type I ES cell clones into 3.5-day C57BL/6 blastocysts using standard techniques (29, 30). Heterozygous null allele mice were obtained by crossing the chimeric males with C57BL/6 or 129Sv females. The heterozygous mice were then intercrossed to obtain α1,2-mannosidase IB-deficient mice. Heterozygous mice on a mixed 129Sv and C57BL/6 background were also backcrossed for eight generations onto the CD-1 background.
**α1,2-Mannosidase IB Gene Ablation**

Southern blots (32). Probes were labeled using the megaprime DNA labeling kit (Amersham Biosciences).

**RT-PCR**—Total RNA was isolated from fetal mouse tissues (E18.5) using the RNeasy Protect Mini Kit (Qiagen). To confirm the absence of exon 2, RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen) with the following α1,2-mannosidase IB primer sets: exon 2-specific primers (5′-AGATTAGAGCTGACCATGAAAGC-3′ and 5′-CTTGATCTCCATGTCCTCTGGATC-3′) and internal control primers flanking exons 11–13. Primers located at both extremities of the open reading frame (5′-GATGACTACCCCAGCGCTGCTG-3′ and 5′-GGACAGCAGATTACCCTGAAAGAG-3′) were also used to amplify the RNA transcripts. These were sequenced to confirm the absence of exon 2 and the presence of a frameshift and stop codon following exon 1.

**Histology**—Fetal and neonatal tissues were fixed in 4 or 10% formalin. Following dehydration with ethanol the tissues were embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin.

Morphometry was performed on images of E18.5 (9–10 lungs) and neonatal (4–5 lungs) hematoxylin and eosin-stained lungs. Images of randomly chosen comparable fields (9–10 per lung) of fetal (E18.5) and neonatal lungs fixed with 2.5% glutaraldehyde in 0.1 M cacodylate at 4 °C for 48 h followed by 1% OsO4 staining. The tissues were dehydrated, embedded in epoxy resin (EMbed 812/Araldite 502 or Epon), cut with a Diatome knife to silver interference color (70–80 nm), and analyzed on a Philips CM100 TEM at 100 kV.

Western Blot Analysis—Fetal lungs (E18.5) were homogenized in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris) supplemented with the protease inhibitor mixture for mammalian tissues (1/500, Sigma). Protein concentration was determined by a Lowry assay. Aliquots containing 35 μg of total protein derived from different lungs were resolved by SDS-PAGE (12% gels, non-reducing for surfactant analysis, reducing for vascular endothelial growth factor (VEGF) analysis), and transferred to polyvinylidene fluoride membranes. The membranes were treated with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 and the avidin-biotin blocking kit (Vector). The blots were then incubated with VEGF (1/1000, A-20, Santa Cruz), Surfactant B (1/1000, Chemicon), or pro-Surfactant C (1/1000, Chemicon) antibodies. The proteins were visualized by ECL with the Elite ABC Vectastain kit (Vector) following binding of biotinylated secondary antibodies (1/10,000, Chemicon). Equal protein loading was confirmed by probing membranes stripped with 2% SDS, 0.1 M β-mercaptoethanol, 62.5 mM Tris, pH 6.8, at 50 °C for 30 min, with actin antibodies (1/1000, Sigma).

**N-Glycan Profiling by MALDI-TOF Mass Spectrometry**—Glycans were isolated from trypsinized detergent extracts of E18.5 brain, thymus, lung, heart, liver, spleen, and kidney by peptide N-glycanase F, permethylated by using the sodium hydroxide procedure, and purified on a Sep-Pak C18 cartridge, as previously described (33). MALDI-MS instrumentation was described previously (34). Derivatized carbohydrate samples were dissolved in methanol/water, 8:2 (v/v), and mixed in a 1:1 ratio with 10 mg/ml 2,5-dihydroxybenzoic acid in 80:20 (v/v) methanol/water. About 1.5-μl aliquots were spotted onto a 100-well sample plate. Angiotensin I, adrenocorticotropic hormone (ACTH) fragment 1–17, ACTH fragment 18–39, and ACTH fragment 7–38 were used for external calibration.

**RESULTS**

Expression of Golgi α1,2-Mannosidases IA, IB, and IC—The developmental and tissue-specific expression of the three mouse Golgi α1,2-mannosidases was investigated by Northern blot analysis (Fig. 1), and in situ hybridization (Fig. 2). Transcripts of α1,2-mannosidase IA (2.7 and 4.8 kb) are expressed at high levels during early post-implantation development (E4.5–E9.5). Similarly, the α1,2-mannosidase IB transcripts (8.2, 6, and 3.6 kb) are also elevated during early embryogenesis (E4.5–E6.5). In contrast, the levels of α1,2-mannosidase IC transcripts (3.4 kb) remain relatively constant throughout embryonic development (Fig. 1A).
In adult mouse tissues, \(\alpha1,2\)-mannosidase IA and IB transcripts display distinct patterns of differential tissue-specific expression (Fig. 1B). \(\alpha1,2\)-mannosidase IA is relatively high in liver, spleen, kidney, stomach, intestine, thymus, uterus, and placenta, whereas \(\alpha1,2\)-mannosidase IB is highly expressed in the testis, thymus, uterus, and placenta. As observed in the embryos, the levels of \(\alpha1,2\)-mannosidase IC are similar in most tissues, with the exception of placenta and liver. The \(\alpha1,2\)-mannosidase IA and IB expression patterns observed in mouse tissues are similar to those reported previously (17, 18).

**In situ** hybridization revealed very high levels of \(\alpha1,2\)-mannosidase IA only in fetal liver, gut, and thymus. In contrast, the \(\alpha1,2\)-mannosidase IB transcripts are strongly expressed in many tissues including the kidney, lung, central nervous system, spleen, thymus, and gut. Transcripts of \(\alpha1,2\)-mannosidase IC exhibit a different pattern of expression with relatively even distribution in many tissues, and strong expression in the kidney and cerebellum (Fig. 2). The observed differences in the developmental expression patterns of \(\alpha1,2\)-mannosidase IA, IB, and IC, as well as their tissue-specific expression suggest that these Golgi enzymes fulfill specialized physiological roles.

**\(\alpha1,2\)-mannosidase IB Gene Ablation**—Considering its prominent expression during embryogenesis, targeted gene ablation of mouse \(\alpha1,2\)-mannosidase IB was performed to elucidate its specific developmental and physiological roles. At the time we undertook these studies it was the only mammalian \(\alpha1,2\)-mannosidase gene whose intron-exon structure had been determined and for which genomic clones were available (14).

Mouse \(\alpha1,2\)-Mannosidase IB is a type II membrane protein encoded by a gene localized on chromosome 3F2 that contains at least 13 exons, and spans a minimum of 80 kb. The protein consists of a cytoplasmic tail (amino acids 1–36), followed by the transmembrane domain, stem region (amino acids 59–170), and a large C-terminal catalytic domain (amino acids 171–641) that is encoded by exons 2–13. A targeting construct inserting floxP sites around exon 2 (amino acids 102–186) of the \(\alpha1,2\)-mannosidase IB gene was prepared using the plox vector (Fig. 3). Cre-mediated excision of the floxed exon causes a frameshift and introduces a stop codon immediately following exon 1, thus producing a null allele. Chimeric mice generated by blastocyst injection of Type I ES cells were bred with C57BL/6 females to obtain heterozygous mice. The heterozygous mice did not display any remarkable phenotype nor any histological abnormalities compared with wild type littersmates.

**Neonatal Lethality of Homozygous Null Mice**—Heterozygous mice (mixed 129Sv and C57BL/6 strain) were intercrossed to determine the effect of systemic loss of \(\alpha1,2\)-mannosidase IB. Postnatal genotyping of the progeny (\(>\)P1) by Southern blot analysis (Fig. 3D) indicate a complete absence of homozygous null mice (Table 1). Therefore, timed pregnancies were set up to collect fetuses the day before birth (E18.5) as well as shortly after birth (\(<\)P0.5). Genotyping of these litters revealed that null fetuses survive until term in utero and comprise 24% of the population. However, null neonates perish within several hours of birth (Table 1). Loss of \(\alpha1,2\)-mannosidase IB expression in null mice was confirmed by RT-PCR analysis performed on total RNA isolated from fetal brain, thymus, lung (Fig. 3E), heart, liver, spleen, and kidney. Homozygous null mouse RNA transcripts amplified by RT-PCR were sequenced to confirm the absence of exon 2 resulting in a frameshift and stop codon immediately following the first exon, thus preventing transla-
tion of the catalytic domain. The α1,2-mannosidase IB gene ablation is also neonatal lethal in mice derived from a second independent ES cell clone, and in the inbred 129Sv and outbred CD-1 strains.

Examination of 18.5-day fetuses did not reveal any obvious differences in gross appearance between null, heterozygote, and wild type mice that could only be identified by genotyping. Initially, null neonates were also indistinguishable from their littermates. However, within several hours of birth they progressively displayed signs of respiratory distress, cyanosis, and perished within 12 h.

α1,2-Mannosidase IB Deficiency Delays Pulmonary Development—Hematoxylin and eosin-stained E18.5 fetal tissues were examined to identify any developmental defects in null mice. Abnormalities were seen only in the lung, and no significant histopathological changes were observed in other tissues. Normal branching morphogenesis is observed in both null and wild type E18.5 lungs (Fig. 4, A and B). However, the α1,2-mannosidase IB-deficient lungs appear less developed than wild type lungs. The cellular and connective tissue components of the interalveolar septa are equivalently thicker and hypercellular relative to wild type fetal lungs (Fig. 4B, arrows indicate comparable regions). Alveolar hemorrhaging is observed in neonates lacking α1,2-mannosidase IB and the lungs display histological features of developmental delay as described for E18.5 lungs (Fig. 4C). Small isolated atelectatic regions are occasionally observed in the null lungs although all null lungs inflate sufficiently to briefly sustain life (4–10 h). After dissection they were shown to have the same buoyancy in fluid as inflated wild type lungs. Morphometric analysis of both E18.5 and neonatal lungs demonstrates that the observed thickening of alveolar septae is statistically significant. The ratio of septae versus the total alveolar cross-sectional area is consistently greater in α1,2-mannosidase IB-deficient mice, with a concomitant decrease in the alveolar air space (Table 2).

The integrity of lung epithelial and endothelial cells in the null mice was verified using different cell-specific markers. Type II pulmonary epithelial cell staining with surfactant B antibodies does not reveal any differences between null and wild type mice (Fig. 5A). Similarly, no differences are observed with tomato lectin (type I cells), and Clara cell protein 26 antibodies (data not shown). Western blot analysis confirmed that type II epithelial cells of null lungs produce normal levels of the processed mature form of surfactant B, and pro-surfactant C (Fig. 5B). In addition, staining of endothelial cells with CD31/PECAM-1 and CD34 (Fig. 5A) indicates normal pulmonary capillary and blood vessel networks in lungs of null neonates. Quantification of vascular networks detected with both of these markers by immunohistochemistry did not reveal any remarkable differences between null and wild type lungs (data not shown). Similar staining patterns of small and large blood vessels are also observed with von Willebrand factor, thrombomodulin, ICAM-1, and laminin antibodies as well as with the lectin GSLB4 (data not shown). Comparable levels of VEGF expression are found in null and wild type lungs by Western blot analysis (Fig. 5B). Furthermore, no remarkable ultrastructural differences in E18.5 and neonatal lungs were observed by transmission electron microscopy.

Pulmonary Glycoprotein Alterations—Fetal and neonatal tissues were analyzed by lectin histochemistry using a panel of 19
Mass spectrometric analysis of the \(N\)-glycans isolated from brain, thymus, lung, heart, liver, spleen, and kidney of E18.5 fetuses shows that deletion of \(\alpha_{1,2}\)-mannosidase IB does not result in gross modification of the tissue \(N\)-glycan repertoire (supplemental Fig. S1). Therefore, only a minute population of glycoproteins is affected by the ablation of Golgi \(\alpha_{1,2}\)-mannosidase IB.

**DISCUSSION**

This work shows that trimming of \(N\)-glycans by Golgi \(\alpha_{1,2}\)-mannosidase IB on a minority of glycoproteins plays a crucial role in pulmonary development essential to mouse neonatal viability. Histopathological findings indicate that atelecasis neonatorum, pulmonary hypoplasia, and alveolar hemorrhage give rise to fatal respiratory distress in Golgi \(\alpha_{1,2}\)-mannosidase IB-deficient neonates. Mouse lung development is a complex process orchestrated by epithelial-mesenchymal and epithelial-endothelial interactions mediated by a variety of growth factors. It commences at E9.5 when the lung bud emerges from the laryngotraheal groove of the foregut into the neighboring mesoderm. Branching morphogenesis gives rise to the conducting airway tree (pseudoglandular stage E9.5–16) and differentiation of epithelial cells lining the airway begins at E14. Capillaries form in the lung parenchyma, and the terminal lung buds dilate as the lungs grow significantly in size during the canalicular stage of development (E16–17). Throughout the final days of gestation distal airspaces form, the mesenchyme becomes thinner, and blood vessels form juxtaposed to the respiratory epithelium (saccular stage E17.5-P5). Postnatally, septae formation within the terminal saccules generate alveoli, thus increasing the surface area for efficient gas exchange (P5–P30) (reviewed in Refs. 35 and 36). The present results show that E18.5 and neonatal \(\alpha_{1,2}\)-mannosidase IB-deficient lung development appears arrested at the canalicular stage of development and is therefore delayed compared with wild type lungs that have reached the saccular stage. Notably the lungs of the null mice contain atypically thick interalveolar septae as well as a concomitant decrease in lung air space. Hence pulmonary immaturity likely elicits the fatal respiratory distress observed in null allele neonates. In addition, postnatal hemorrhaging restricted to distal pulmonary regions suggests that the integrity of bronchiolar or alveolar sac walls is compromised.

**FIGURE 3.** Targeted disruption of the mouse \(\alpha_{1,2}\)-mannosidase IB gene. A, \(\alpha_{1,2}\)-mannosidase IB targeting construct. Exon 2 is floxed in the construct prepared using the pflox vector containing the tk and neo selectable markers. The genomic DNA highlighted by pink solid lines was inserted into the pflox vector as indicated by the stippled lines, using restriction enzyme sites shown in pink. Blue numbered boxes indicate exons 2–4, red bars labeled \(L\) represent loxP sites, and enzyme sites are denoted: \(B\), BamHI; \(Bg\), BglII; \(E\), EcoRI; \(N\), NotI; \(S\), Spel; \(Sa\), Sali; \(Sc\), SacII; \(St\), Stul; \(X\), XhoI. 

B, targeted allele (ManIB\(^{TNf}\)) and location of genomic probes exterior to sites of homologous recombination. Restriction enzyme sites in parentheses were lost upon ligation of genomic DNA into the targeting vector.

C, null (ManIB\(^{-}\)) and floxed (ManIB\(^{f}\)) alleles generated by transient transfection of homologous recombinants (ManIB\(^{TNf}\)) with Cre recombinase and FIAU selection of clones containing deletion of loxP-flanked DNA. 

D, Southern blot analysis of restriction endonuclease-digested DNA isolated from ES cells and mice containing a targeted (TNf), or null allele hybridized with genomic probes. 

E, RT-PCR analysis of E18.5 null and wild type lung RNA using primers to amplify exon 2 (207-bp amplicon) and an internal control spanning exons 11–13 (401-bp amplicon), as described under “Experimental Procedures.”

**A**

ManIB\(^{WT}\)

\(\text{pflox}\)

\(\text{loxP}\)

\(\text{tk}\)

\(\text{neo}\)

\(\text{flxed}\)

\(\text{ManIB\(^{-}\)}\)

\(\text{ManIB\(^{TNf}\)}\)

\(\text{ManIB\(^{f}\)}\)

**B**

**C**

**D**

**E**
**α1,2-Mannosidase IB Gene Ablation**

**TABLE 1**

Genotype of progeny arising from intercrossing heterozygous (−/+ ) mice

| Genotype | E18.5 | <P0.5 | >P1 |
|----------|-------|-------|-----|
| +/+      | 28%   | 21%   | 32% |
| −/+      | 48%   | 52%   | 68% |
| −/−      | 24%   | 27%   | _b_ |
| n       | 361   | 161   | 754 |

 superficially. C57BL/6 and 129 mixed background.

 2 The number of mice analyzed for each genotype is indicated in parentheses.

 3 Differences between age-matched mean ratios were determined to be significant by the Student’s t test (p < 0.01).

mised in the developmentally delayed lungs. Leakage of blood into the alveolar sacs would further impede oxygen exchange and progressively exacerbate neonatal respiratory distress. Thus Golgi α1,2-mannosidase IB-mediated N-glycan biosynthesis contributes to the timely development of thin saccular walls and epithelial/endothelial barriers capable of supporting increased pulmonary arterial blood circulation postnatally, and the transition to sustained breathing after birth.

The only significant histochemical difference observed in null E18.5 fetuses and neonates is the loss of L-PHA, E-PHA, and D. stramonium reactive glycoproteins in a subpopulation of epithelial cells lining the bronchioles. Alteration of a restricted set of pulmonary glycoproteins within this subpopulation of pulmonary cells indicates that typical N-glycan maturation proceeds normally in the vast majority of cells of the α1,2-mannosidase IB null mice due to the presence of the other Golgi α1,2-mannosidases. Remarkably, analysis of N-glycans in Golgi α1,2-mannosidase IB-deficient tissues by mass spectrometry did not detect any significant global alterations in complex N-glycans, confirming that the molecular mechanisms underlying the lethal phenotype of the α1,2-mannosidase IB null mouse is triggered by N-glycan alterations in a relatively minute subpopulation of glycoproteins. This is the first example of such a specific and restricted effect on N-glycan biosynthesis caused by inactivation of an early processing enzyme. The specific role of α1,2-mannosidase IB might be due to a restricted specificity for certain glycoproteins and/or to its exclusive expression in...
The observation that eliminating Golgi α1,2-mannosidase IB causes a lethal specific lung phenotype with little change in global N-glycosylation is consistent with recent studies indicating that major physiological effects can be caused by altered glycosylation of individual glycoproteins induced by glycosyltransferase gene ablations. In one study, a pulmonary defect similar to human emphysema was observed due to inactivation of FucTIII causing the loss of core α1,6-fucose addition to the transforming growth factor-β1 receptor (39). The importance of fucosylation in transforming growth factor-β1 receptor regulation was established by rescuing transforming growth factor-β1 signaling in fibroblasts derived from null mice by transfection of the fucosyltransferase, as well as by correction of the pulmonary defects following postnatal injection of exogenous transforming growth factor-β1. Another report showed that Type II diabetes can be elicited by ablation of the Mga4a gene encoding N-acetylglucosaminyltransferase IV. In this case altered glycosylation of the glucose transporter affects its cell surface localization in pancreatic β cells, thereby effectively blocking its function (40). Although the specific glycoproteins altered by eliminating Golgi α1,2-mannosidase IB remain to be identified, the present results indicate that affecting glycosylation of a few glycoproteins can have a dramatic effect on lung development and function. These essential glycoproteins may be expressed within the developing lung, but it is also possible that cryptic defective glycosylation of molecules at remote sites could impact lung development leading to the lethal hemorrhage.

The pulmonary developmental delay observed in the Golgi α1,2-mannosidase IB-deficient mouse differs from any of the previously reported lung phenotypes caused by a variety of targeted gene ablations that, for example, interfere with branching morphogenesis, vascularization, epithelial cell maturation, surfactant B production, etc. (reviewed in Refs. 41–43). The phenotype of α1,2-mannosidase IB-deficient mice is clearly different because the lungs display normal expression of epithelial and endothelial cell markers including surfactant B, pro-surfactant C, CD31/PECAM-1, CD34, and VEGF. Further characterization of the mouse lung defect will provide novel insight into lung development that may be relevant to understanding the causes of idiopathic human neonatal pulmonary hemorrhage occurring in some premature infants (Refs. 44–47 and references cited therein). There is an increasing number of multisystemic human genetic diseases known as congenital diseases of glycosylation characterized by hypoglycosylation of glycoproteins due to mutations in different glycosylation enzymes (4,


**α1,2-Mannosidase IB Gene Ablation**

48. The phenotype of the Golgi α1,2-mannosidase IB-deficient mouse suggests that some of the human neonatal lung problems of unknown etiology might be due to genetic defects in α1,2-mannosidases involved in N-glycan maturation.

**Acknowledgments**—We thank Dr. Michel Tremblay for advice on this project, Dr. Jamey D. Marth for generously providing the pflox vector, Daniel Chui for guidance in preparing the targeting construct, the McGill Transgenic Core Facility for blastocyst injection services, and Craig Fleming for histological assistance.

**REFERENCES**

1. Herscovics, A. (2001) *Biochimie (Paris)* 83, 757–762
2. Metzler, M., Gertz, A., Sarkar, M., Schachter, H., Schrader, J. W., and Marth, J. D. (1994) *EMBO J.* 13, 2056–2065
3. Ioffe, E., and Stanley, P. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 728–732
4. Jaeken, J., and Carchon, H. (2004) *Curr. Opin. Pediatr.* 16, 434–439
5. Camirand, A., Heysen, A., Grondin, B., and Herscovics, A. (1991) *J. Biol. Chem.* 266, 15120–15127
6. Gonzalez, D. S., Karaveg, K., Vandersall-Nairn, A. S., Lal, A., and Moremen, K. W. (2004) *J. Biol. Chem.* 279, 29774–29786
7. Lal, A., Pang, P., Kalelkar, S., Romero, P. A., Herscovics, A., and Moremen, K. W. (1998) *Glycobiology* 8, 981–995
8. Ioffe, E., Liu, Y., and Stanley, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11041–11046
9. Hato, M., Nakagawa, H., Kurogochi, M., Akama, T. O., Marth, J. D., Fukuda, M. N. (2002) *Science* 295, 124–127
10. Akama, T. O., Nakagawa, H., Wong, N. K., Sutton-Smith, M., Dell, A., Morris, H. R., Nakayama, I., Nishimura, S., Pai, A., Moremen, K. W., Marth, J. D., and Fukuda, M. N. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 8983–8988
11. Seidah, N., Benjannet, S., Wickham, L., Marcinkiewicz, J., Jasmin, S., Stifani, S., Basak, A., Prat, A., and Chretien, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 928–933
12. Essalmani, R., Hamelin, J., Marcinkiewicz, J., Chamberland, A., Mbikay, M., Chretien, M., Seidah, N., and Prat, A. (2006) *Mol. Cell. Biol.* 26, 354–361
13. Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, 2 ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Wasserman, P. M., and DePamphilis, M. L. (eds) (1993) *Methods in Enzymology: Guides to Techniques in Mouse Development*, Vol. 225, Academic Press Inc., New York
15. Prietel, J. J., Sakar, M., Schachter, H., and Marth, J. D. (1997) *Glycobiology* 7, 45–56
16. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. A., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) in *Current Protocols in Molecular Biology* (Franzen, K., ed) Vol. 1, John Wiley and Sons, Inc., New York
17. Sutton-Smith, M., Morris, H. R., and Dell, A. (2000) *Tetrahedron Asymmetry* 11, 363–369
18. Kui Wong, N., Easton, R. L., Panico, M., Sutton-Smith, M., Morrison, J. C., Lattanzio, F. A., Morris, H. R., Clark, G. F., Dell, A., and Patankar, M. S. (2003) *J. Biol. Chem.* 278, 28619–28634
19. Cardoso, W. V. (2000) *Dev. Dyn.* 219, 121–130
20. Roth-Kleiner, M., and Post, M. (2003) *Biol. Neonat.* 84, 83–88
21. Ioffe, E., Liu, Y., and Stanley, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11041–11046
22. Hato, M., Nakagawa, H., Kurogochi, M., Akama, T. O., Marth, J. D., Fukuda, M. N., and Nishimura, S. I. (2006) *Mol. Cell. Proteomics* 5, 2146–2157
23. Wang, X., Inoue, S., Gu, J., Miyoshi, E., Noda, K., Li, W., Mizuno-Horikawa, Y., Nakano, M., Asahi, M., Takahashi, M., Uozumi, N., Ihara, S., Lee, S., Ikeda, Y., Yamaguchi, Y., Aze, Y., Tomiyama, Y., Fujii, J., Suzuki, K., Kondo, A., Shapiro, S., Lopez-Otin, C., Kuwaki, T., Okabe, M., Honke, K., and Taniguchi, N. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 15791–15796
24. Ohtsubo, K., Takamatsu, S., Minowa, M. T., Yoshida, A., Takeuchi, M., and Marth, J. D. (2005) *Cell* 123, 1307–1321
25. Warburton, D., Schwartz, M., Teft, D., Fores-Delgado, G., Anderson, K., and Cardoso, W. V. (2000) *Mech. Dev.* 92, 55–81
26. Costa, R. H., Kalinichenko, V. V., and Lim, L. (2001) *Am. J. Physiol.* 280, L823–L838
27. deVello, D. (2004) *Semin. Neonat.* 9, 311–329
28. Berger, T. M., Allred, E. N., and Van Marter, L. J. (2000) *J. Perinatol.* 15, 490–498
29. Cole, V. A., Normand, I. C., Reynolds, E. O., and Rivers, R. P. (1973) *Pediatrics* 51, 175–186
30. Kalinichenko, V. V., Lim, L., Stolz, D. B., Shin, B., Rausa, F. M., Clark, J., Whitsett, J. A., Watkins, S. C., and Costa, R. H. (2001) *Dev. Biol.* 235, 489–506
31. Rezkalla, M. A., and Simmons, J. L. (1995) *S. J. Med.* 48, 79–85
32. Freeze, H. H., and Aebi, M. (2005) *Curr. Opin. Struct. Biol.* 15, 490–498