CHARACTERISATION OF COMPENSATORY ROLES OF α-MANNOSIDASE II AND α-MANNOSIDASE IIX IN N-GLYCANS PROCESSING

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ABSTRACT. *The potential and relative role of α-mannosidase IIX (MIIX) in complex N-glycan biosynthesis in vivo, was studied by generating and characterizing mice lacking Man2a1 (MII knockout), Man2a2 (MIIX knockout) and both Man2a1and Man2a2 (double knockout). Pathological Abnormalities in Double-Null Neonates and Embryos were observed in liver and lung. Morphological analysis of double-nulls died between embryonic days 15.5 and 18.5, but most survived until shortly after birth and or died before postnatal day 2 due to respiratory failure, which represents a more severe phenotype than that seen in single-nulls for either gene. The ability of the MII, MIIX and double null mice generating complex type N-glycans were characterized by using a newly developed methodology based on high sensitivity mass spectrometric techniques including MALDI-MS, ESI-MS, FAB-MS, CAD MS/MS and GC-MS techniques complemented by a wide range of chemical and enzymatic methodologies. The MIIX knockout mouse serum and embryo resulted in a significant reduction in complex type N-glycans. Whilst, the double depletion of MII and MIIX genes from mouse serum and embryo showed a complete disappearance of complex type structures concurrent with the appearance of mono-and bi-antennary hybrid type N-glycans with and without core fucosylation. These observations and the absence of complex type N-glycans in the double knockout MII and MIIX genes reveals that MIIX is the only alternative α-mannosidase II-like enzyme that is active throughout embryogenesis, thereby suggesting an alternative pathway for N-glycan processing.*

KEYWORDS. N-glycan processing, Mass spectrometry, -Mannosidase II, α-Mannosidase IIX

INTRODUCTION

N-glycosylation is the major form of posttranslational modification of newly synthesized proteins through the secretory pathway. The major biosynthetic steps for *N*-glycans in vertebrates have been established (Konford *et al.,* 1985; Schacheter 1991). A key conversion of high mannose to complex-type oligosaccharides occurs in the medial Golgi, where GlcNAc-transferase I (GlcNAc-TI) adds a GlcNAc residue to form a hybrid-type *N*-glycan, GlcNAc1Man5GlcNAc2 (Schachter *et al*., 1983)).

Golgi α-mannosidase II (MII) then removes two mannosyl residues to form GlcNAc1Man3GlcNAc2 (Tulsiani *et al*., 1982; Moreman *et al.,* 1994), which is further modified by GlcNAc-transferase II (GlcNAc-TII) (Harpaz *et al.,* 1980) to form GlcNAc2Man3GlcNAc2, the precursor of complex-type *N* glycans. Although MII catalyzes the step after GlcNAc-TI and before GlcNAc-TII (Konford *et al.,* 1985; Schacheter, 1991), MII-null mice are born and are apparently normal except for dyserythropoiesis, causing a phenotype similar to that observed in human congenital dyserythropietic anemia type II or hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS) (Chui *et al.,* 1997). Furthermore, MII-nulls synthesize complex-type *N*-glycans in many tissues, despite the absence of MII enzyme activity. These findings led to the proposal of an alternative pathway for the production of complex *N*-glycans (Chui *et al.,* 1997).

Two candidate enzymes could have been possible to function in this pathway; one has been reported as a cobalt-dependent broad-specificity -mannosidase activity in rat liver microsomes (Bonay *et al.,* 1991; Bonay *et al.,* 1992), and the other is -mannosidase IIX. Human MIIX (Misago *et al.,* 1995) is the product of the *MAN2A2* gene and is homologous to human MII, which is encoded by *MAN2A1*. It has been suggested from previous study that human *MAN2A2* is catalytically active and plays a role in *N*-glycan biosynthesis (Misago *et al.,* 1995; Oh-Eda *et al.,* 2001; Akama *et al.,* 2002). When mouse *Man2a2*, the orthologue of human *MAN2A2*, was disrupted, -nulls were apparently normal, except that mutant males were subfertile (Akama *et al.,* 2002). In the mouse testis, *N*-linked carbohydrate structures were altered compared to wild-type mice, suggesting that MIIX also acts to process *N*-glycans. Nonetheless, the role of MIIX in cells other than spermatogenic germ cells remains unclear. Therefore, in this study, mice lacking both *Man2a1* and *Man2a2* were generated and characterized to determine the potential and relative role of MIIX in complex *N*-glycan biosynthesis *in vivo*.

MATERIALS AND METHODS

Production of Man2a1(+/-)/Man2a2(+/-) Double-Null Mice.

Man2a1(+/-) (13) and *Man2a2*(+/-) mice (18) were mated. Resulting *Man2a1*(+/-)/*Man2a2*(+/-) mice were then crossed to obtain double-null animals. For genotyping, PCR analysis of each allele was performed by using allele-specific primers with tail biopsy DNA as a template. To analyze the survival rate of double-null mice at embryonic and neonatal stages, *Man2a1*(+/-)/*Man2a2*(+/-) mice were crossed, and embryos were recovered at defined time points. Embryos or isolated tissues were either fixed in 4% paraformaldehyde in PBS or stored at -80°C until use.

Construction of Expression Vectors for MII and MIIX Enzymes.
DNA fragments encoding full-length or soluble forms of mouse MII and MIIX were amplified from a mouse brain cDNA library (BD Biosciences, Palo Alto, CA) by PCR. After confirmation of the sequences, DNA fragments encoding full-length enzymes were subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen). cDNA fragments encoding soluble forms of enzymes were subcloned into pcDNA-HSH.

MALDI-TOF Analysis of Permethylated N-Glycans.
Embryos were homogenized on ice in extraction buffer containing 0.5% wt/vol SDS/0.1 M Tris/HCl, pH 7.4. After dialysis and lyophilization, extracts were reduced and carboxymethylated followed by sequential tryptic and peptide *N*-glycosidase F digestion and Sep-Pak purification.MALDI-TOF data were acquired by using a Voyager-DE STR mass spectrometer (Applied Biosystems) in the reflectron mode with delayed extraction. Permethylated samples were dissolved in 10 µl of methanol, and 1 µl of each dissolved sample was premixed with 1 µl of matrix (2,5-dihydrobenzoic acid) before loading onto a metal plate.

RESULTS AND DICUSSION

Generation of MII/MIIX Double-Mutant Mice.
The genes encoding MII (*Man2a1*, chromosome 17E1) and MIIX (*Man2a2*, chromo-some 7D1) are located on different chromosomes in the mouse, which enabled us to obtain mice lacking MII and MIIX genes by crossing double heterozygote *Man2a1*(+/-)/*Man2a2*(+/-) mice. However, we observed that most of the double-nulls died shortly after birth, and very few survived up to 3 weeks. Thus, in order to be more efficiently produce double-null pups, *Man2a1*(+/-)/*Man2a2*(+/-) mice were crossed; double-null pups resulting from this cross also showed early lethality. Genotyping of embryos from such crosses showed that almost all double-nulls survived until E15, some double-nulls died between E15 and the day of birth (E18), and most double nulls died soon after birth or before postnatal day 2 (Figure 1*A*). We also observed that many double-null neonates actively gasped for air at birth but failed to breathe properly and died, suggesting that lethality of MII/MIIX double-null neonates is because of respiratory failure.

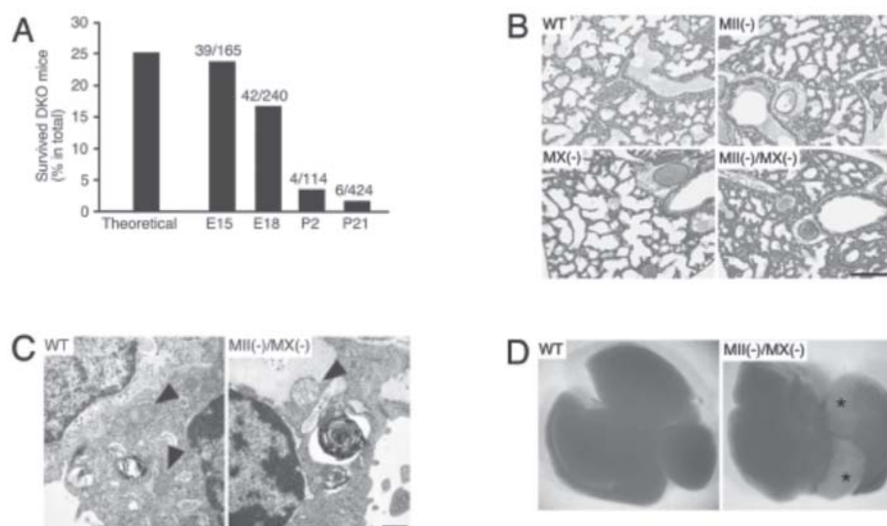

Figure 1. Survival of MII/MIIX double-nulls during embryonic and postnatal development and morphological analysis of double-nulls. (*A*) Survival rate of MII/MIIX double-nulls during embryonic and postnatal development. *Man2a1*(+/-)/*Man2a2*(+/-) mice were crossed, and embryos obtained from pregnant female mice and postnatal pups were genotyped. Each bar represents the relative numbers of surviving double-null embryos and neonates per total offspring. (*B*) Histological observation of neonatal mouse lung. Hematoxylin_eosin (H&E) staining of paraffin-embedded tissue sections demonstrated that the double-null neonatal lungs have less air space and thicker alveolar septa. (Scale bar, 200 _m.) (*C*) Electron micrographs of alveolar type II pneumocytes from wild-type and MII/MIIX double-null animals. Note the presence of large vacuoles and enlarged mitochondria in the double-nulls (mitochondria in both pictures are indicated by arrowheads). (Scale bar, 500 nm.) (*D*) Macroscopic observation of neonatal livers. Apparent abnormalities in the double-null liver are marked by asterisks. (*E*) Apoptosis analysis of cells from MII/MIIX double-null liver. Paraffin-embedded section of double-null E15 embryo displayed signs of massive apoptosis in liver, correlated with palerH&E staining. (*F*) Electron micrographs of hepatocytes from wild-type and MII/MIIX double-null embryos at E15. Mitochondria in both pictures are indicated by arrowheads. Note that the double-null hepatocyte contains large vacuoles and enlarged mitochondria, similar to observations of lung type II pneumocytes (*C*). (Scale bar, 2 _m.).

Pathological Abnormalities in Double-Null Neonates and Embryos.

Histological analysis indicated that lung tissue of double-null neonates had less alveolar air space by comparison to lung tissue from wild-type mice or mice mutant in either MII or MIIX (Figure 1*B*). The pulmonary epithelial cell layer of double-nulls appeared thicker than that of either wild type or single-nulls, consistent with a failure of alveolar expansion in double-nulls. These morphological characteristics support the hypothesis that respiratory distress was the cause of lethality in double-null neonates. Electron microscopy of type II pneumocytes, which produce multilamellar bodies composed of surfactant proteins that prevent tissue collapse during respiration, showed abnormal vacuole formation and enlarged mitochondria in double-null animals (Figure 1*C*). The livers of double-nulls were also frequently damaged in neonates and E15 embryos (Figure 1*D*). Apoptotic signals were increased in damaged areas of the double-null liver from E15 embryos, indicating increased cell death (Figure 1*E*). Electron microscopy of the E15 liver from a double-null mouse showed abnormal vacuoles and enlarged mitochondria (Figure 1*F*), characteristics also seen in the lungs of double-nulls (Figure 1*C*). Similar abnormal vacuoles and enlarged mitochondria were seen in proximal tubule cells of the double-null kidney. However, these abnormal morphologies were not apparent in other tissues, such as heart, small intestine, and pancreas, in double-null mice, suggesting that the appearance of vacuoles and enlarged mitochondria is cell-type dependent.

Despite such low expression levels, the existence of MIIX activity in tissues other than testis is evidenced by the ability of those tissues derived from the MII-null mouse to synthesize complex-type *N*-glycans. It is likely that, in many cell types, MII rather than MIIX is the major contributor to *N*-glycan processing.

Absence of Complex-Type N-Glycans in Double-Null Embryos.

MALDI-TOF MS (Figure 2) showed that the *N*-glycans from both wild-type and MIIX-null mice are mainly complex-type (Figure 2A and Figure 2B). They also contained high-mannose-type but not significant levels of hybrid-type *N*-glycans. In contrast, MII-null embryos showed high levels of hybrid-type *N*-glycans and reduced levels of complex-type *N*-glycans (Figure 2C), whereas in double-null embryos, signals for hybrid-type *N*-glycans were significantly increased, and complex-type *N*-glycans had completely disappeared (Figure 2D). Based on these results presented in Figure 2, we conclude that MIIX functions in *N*-glycan processing *in vivo* in the mouse, and that lack of both MII and MIIX leads to arrest of *N*-glycan processing at hybrid-type structures.

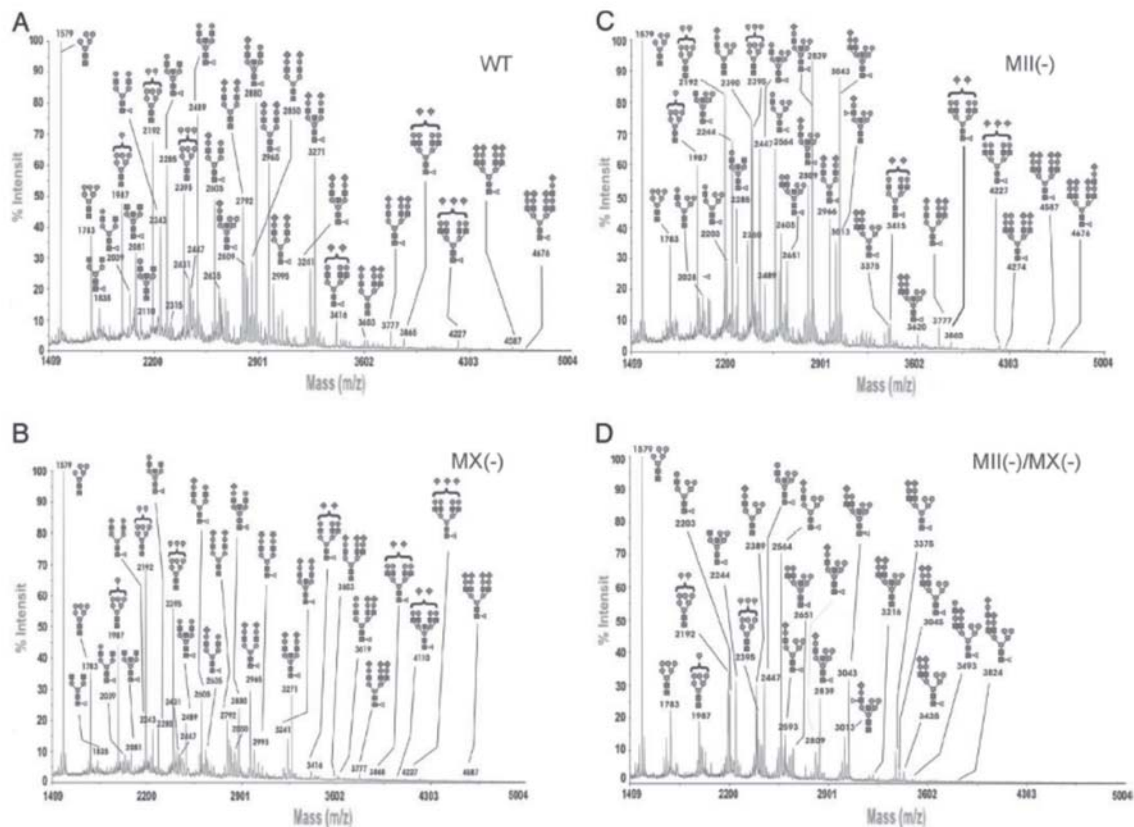

Figure 2. MALDI-TOF analysis of *N*-glycans from E15 embryos. (*A*) Wild type, (*B*) MIIX-null, (*C*) MII-null, and (*D*) MII_MIIX double-null. *N*-glycans were released from embryo homogenates by peptide *N*-glycosidase F digestion and were permethylated before MALDI-TOF analysis. Annotations are based on compositional information provided by MALDI molecular weights, complemented by collisional activation tandem MS and linkage analysis experiments (data not shown).

The data presented here indicate an absence of complex-type *N*-glycans in MII/MIIX double-null mice (Figure 2), demonstrating that MIIX is the only enzyme able to compensate for the absence of the MII step in *N*-glycan processing *in vivo*.

Based on all data obtained in this study, a proposed *N*-glycan processing pathway is shown in Figure 3, in which this pathway clearly indicates the only two 3,6-mannosidases capable of providing a processing route to complex-type *N*-glycan structures in animal systems.

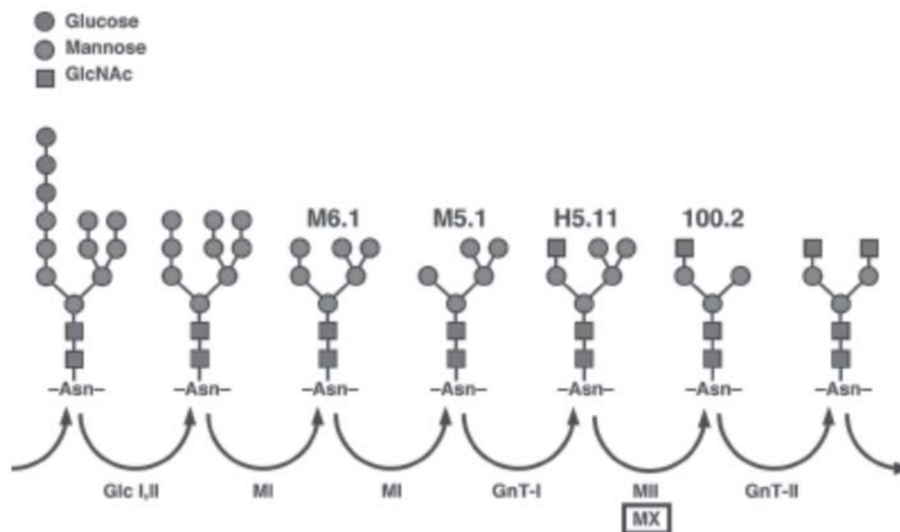

Figure 3. The *N*-glycan processing pathway, including MIIX. This study demonstrates that MII and MIIX are isozymes hydrolyzing the hybrid-type N-glycan structures, and that either enzyme is required for formation of complex-type *N*-glycans.

CONCLUSION

These findings have shown that *N*-glycan maturation, extension and the formation of complex-type structures are extremely important for normal mouse development because the double-null mice that completely lack complex-type *N*-glycans die shortly after birth. In addition, MIIX was demonstrated to be the only enzyme functionally very similar to MII. No other enzymes can rescue the loss of complex type *N*-glycan formation *in vivo*. Thus, an alternative bypass pathway was proposed in MII-null animals in which MIIX acts as the compensating enzyme in N-glycan processing. This study also excludes the likelihood of an alternative processing pathway catalyzed by a different enzyme capable of bypassing this critical processing step in MII/MIIX double-null mice.

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