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Alpha-Mannosidase-II Deficiency Results in Dyserythropoiesis and Unveils an Alternate Pathway in Oligosaccharide Biosynthesis

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Summary

Alpha-mannosidase-II (αM-II) catalyzes the first committed step in the biosynthesis of complex asparagine-linked (N-linked) oligosaccharides (N-glycans). Genetic deficiency of αM-II should abolish complex N-glycan production as reportedly does inhibition of αM-II by swainsonine. We find that mice lacking a functional αM-II gene develop a dyserythropoietic anemia concurrent with loss of erythrocyte complex N-glycans. Unexpectedly, nonerythroid cell types continued to produce complex N-glycans by an alternate pathway comprising a distinct α-mannosidase. These studies reveal cell-type-specific variations in N-linked oligosaccharide biosynthesis and an essential role for αM-II in the formation of erythroid complex N-glycans. αM-II deficiency elicits a phenotype in mice that correlates with human congenital dyserythropoietic anemia type II.

Introduction

Vertebrate cell surfaces are covered with a diverse and dynamic repertoire of asparagine-linked (N-linked) oligosaccharides (also termed N-glycans, indicating linkage to underlying protein). Complex N-glycans are the most abundant type found on the cell surface, and alpha-mannosidase-II (αM-II) acts as a key enzyme in their biosynthesis by catalyzing the first committed step in the conversion of hybrid to complex forms (Kornfeld and Kornfeld, 1985; Schachter, 1991; Figure 1). In the Golgi apparatus, αM-II cleaves two mannose residues attached in α3 and α6 linkages from the hybrid N-glycan $GlcNAc_1Man_5GlcNAc_2$-Asn, thereby producing a processed hybrid that is also the specific substrate of GlcNAc-TII (Harpaz and Schachter, 1980; Tulsiani et al., 1982; Moremen et al., 1994; Figure 1). This role is further supported from studies of the Ric15 BHK cell line, in which a reduction in αM-II activity occurs with an attenuation of complex N-glycan production (Hughes and Feeney, 1986). An exogenous inhibitor of αM-II activity known as swainsonine is found in plants of the genus Swainsona (Elbein et al., 1981; Tulsiani et al., 1982). Ingestion by vertebrates produces a disease known as "locoism," biochemically similar to α-mannosidosis and associated with aberrant behavior, male sterility, cytoplasmic vacuolation, and the accumulation of hybrid N-glycans in the brain and other tissues (Dorling et al., 1978; Colgate et al., 1979; Tulisani et al., 1988). In animal studies with the purified alkaloid, the effects of locoweed ingestion appear to be due to swainsonine (Tulsiani et al., 1984, 1988). However, a role for αM-II deficiency in these responses is not clear, since swainsonine is also a potent inhibitor of the lysosomal α-mannosidase, which functions in N-glycan catabolism (Dorling et al., 1980; Tulsiani et al., 1982; Tulsiani and Touster, 1987).

Human deficiency of αM-II has been reported in one case of congenital dyserythropoietic anemia (CDA) type II, also known as HEMPAS (hereditary erythroblastic multinuclearity with a positive acidified serum-lysis test) disease (Crookston et al., 1969; Fukuda et al., 1990). CDA type II/HEMPAS is inherited in an autosomal recessive manner, with patients developing mild-to-severe anemia associated with splenomegaly and marrow erythroplasia in the presence of multinucleated erythroblasts (reviewed in Fukuda, 1993). Most patients live a normal lifespan without neurologic involvement, although complications, including hepatomegaly with cirrhosis, hemosiderosis, gallstones, and diabetes, frequently develop. CDA type II erythrocytes commonly exhibit plasma membrane abnormalities with loss of complex N-linked oligosaccharides from glycoproteins band 3 (*AE1*) and band 4.5 (*GLUT1*) (Baines et al., 1982; Scartezzini et al., 1982; Fukuda et al., 1984, 1992). However, the clinical diagnosis of CDA type II encompasses heterogenic symptoms with only one reported case thus far associated with αM-II deficiency, in which the patient retained only 10% of normal αM-II levels with an unresolved genetic defect.

The biological role of αM-II is further puzzling, considering its pivotal position in complex N-glycan production and the relatively mild phenotype of CDA type II when compared to GlcNAc-TII deficiency in the next biosynthetic step. Studies have linked inactivating mutations in the *MGAT2* gene to human carbohydrate-deficient glycoprotein syndrome (CDGS) type II (Tan et al., 1996). This autosomal recessive disease presents severe symptoms early with children exhibiting failure to thrive, dysmorphic features, severe mental retardation, and susceptibilty to multiple infections (Jaeken et al., 1994; Charuk et al., 1995). In order to understand how αM-II functions in mammalian physiology and in N-glycan diversification, it was necessary to inactivate the *αM-II* gene in the mouse germline. We report that mice lacking a functional *αM-II* allele develop a dyserythropoietic anemia similar to CDA type II, with production of abnormal erythrocytes lacking complex N-glycans. Unexpectedly, complex N-glycan production continued among nonerythroid cell types in the absence of αM-II activity by an alternate biosynthetic pathway.

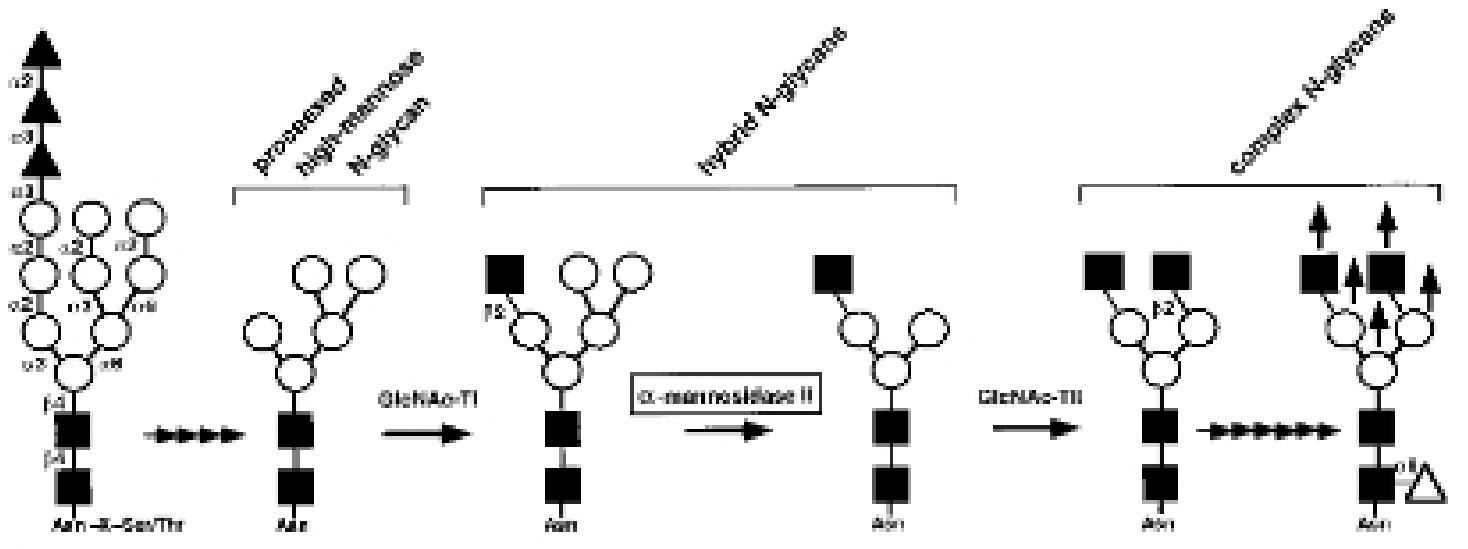

Figure 1. Asparagine-Linked Oligosaccharide Biosynthesis

The depicted oligosaccharide precursor is transferred from lipid to asparagines on nascent peptides in the endoplasmic reticulum prior to glucosidase and α1–2 mannosidase trimming. In the medial Golgi, *Mgat1*-encoded GlcNAc-TI initiates hybrid N-glycan synthesis. Conversion to complex N-glycans requires α-mannosidase-II to generate the processed hybrid N-glycan that is also the substrate of GlcNAc-TII. Anomeric linkage types are denoted. Closed triangles represent glucose; open circles, mannose; closed squares, N-acetylglucosamine. Addition of fucose (open triangle) may occur earlier than indicated. Multi-antennary complex N-glycans result from other branching reactions (arrows).

Results

αM-II Gene Inactivation in Embryonic Stem Cells and Mice

αM-II is a type II transmembrane protein of 1150 amino acids encoded by a single gene locus in studied vertebrates (Moremen and Robbins, 1991; Moremen et al., 1994). A mouse genomic clone that contained an exon representing a portion of the lumenal "stem" domain of αM-II was isolated. As sequence analysis indicated that excision of this exon would create a translational frameshift mutation, a gene targeting construct was produced that would permit excision of this exon and removal of the marker genes by Cre recombinase (Figure 2A; reviewed in Marth, 1996). G418 resistant embryonic stem cell clones were analyzed for homologous recombination events, producing a modified *αM-II* locus bearing three *loxP* sites as required to produce systemic (Δ) and conditional (F) mutations (Figures 2B and 2C). Chimeric mice generated from ES clones 34–107 and 31–107 were bred to C57BL/6 mates to produce mice heterozygous for $\alpha M\text{-}II^{\Delta}$ or $\alpha M\text{-}II^{F}$ alleles, respectively. Offspring homozygous for either allele were obtained at normal frequencies, and those bearing $\alpha M\text{-}II^{\Delta}$ alleles were further analyzed.

Mice Homozygous for the $\alpha M\text{-}II^{\Delta}$ Allele Lack αM-II Enzyme Activity

αM-II activity can be specifically determined using the fluorescent substrate $GlcNAc_1Man_5GlcNAc$-pyridylamine (-PA). Release of mannose alters the chromatographic mobility of this molecule in a defined manner. αM-II has been immunolocalized to the early Golgi apparatus in most cell types (Moremen et al., 1994), and Golgi fractions from genotyped mouse tissues were examined for αM-II activity by a 2 to 24 hr incubation with substrate in the presence or absence of swainsonine. αM-II activity was present in wild-type samples with the generation of the cleaved products $GlcNAc_1Man_4GlcNAc$-PA and $GlcNAc_1Man_3GlcNAc$-PA, but was absent from extracts derived from $\alpha MII^{\Delta}/\alpha M\text{-}II^{\Delta}$ mice or extracts treated with swainsonine (Figures 3A and 3B; data not shown). Additionally, an antibody specific for αM-II failed to immunoprecipitate αM-II activity from total cell extracts derived from $\alpha MII^{\Delta}/\alpha M\text{-}II^{\Delta}$ samples (data not shown). These experiments demonstrated that the exon deletion produced in the *αM-II* gene ($\alpha M\text{-}II^{\Delta}$) results in a complete loss of enzyme activity and is thus a genetic null allele.

Mice Lacking a Functional *αM-II* Allele Exhibit Splenomegaly Associated with Dyserythropoietic Anemia

Mice lacking a functional *αM-II* allele did not display noticable neurologic or behavioral symptoms and bred normally with either wild-type or homozygous-null mates. Histologic findings of the liver, brain, kidney, lymph nodes, heart, lung, and pancreas were unremarkable. Leukocyte development, morphology, circulation, and colonization of secondary lymphoid organs also appeared normal in αM-II-deficient mice (data not shown). However, analyses of peripheral blood indicated high levels of reticulocytes and significant variations in erythrocyte cell size (anisocytosis) (Figure 4A). αM-II-deficient mice of all ages invariably exhibited a marked splenomegaly (Figure 4B). Additionally, detailed hematologic analyses revealed a mild-to-moderate anemia, accompanied by a marked anisocytosis, reduced osmotic fragility, indicating a likely increase in the surface area to volume ratio, reticulocytosis involving up to 30% of circulating red blood cells, and increased levels of the i-antigen (Figures 4C-4G). The αM-II-null bone marrow exhibited normal cellularity but an increase in erythroblasts (Figure 4H). Anisocytosis, reduced osmotic fragility, increased i-antigen levels, and erythroblast hyperplasia were observed in αM-II-null mice of all ages, whereas the severity of the anemia and reticulocytosis increased with age.

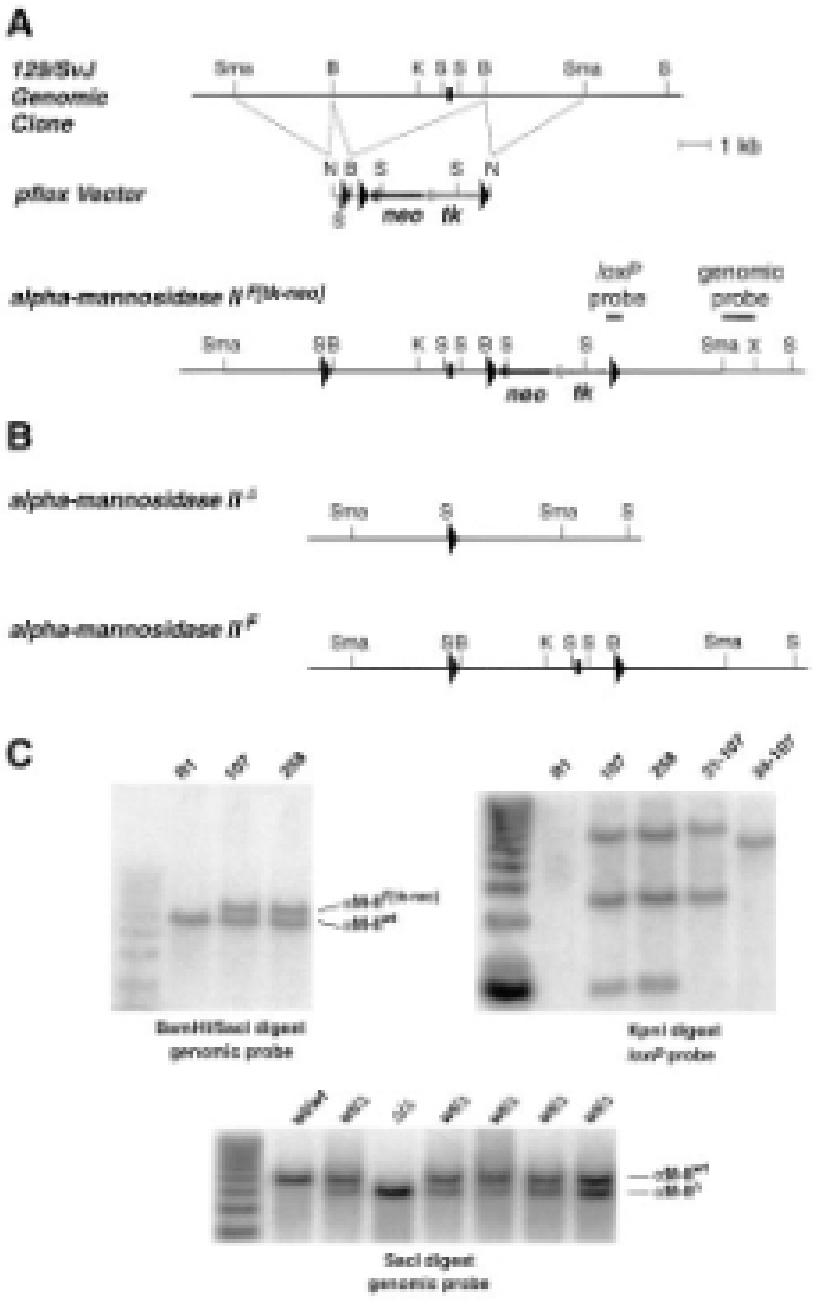

Figure 2. αM-II Gene Mutagenesis in Embryonic Stem Cells and Mice

(A) A mouse genomic clone of αM-II bearing a single exon (closed box) was isolated and used in constructing the αM-II gene targeting vector with pflox as indicated. Homologous recombination with wild-type embryonic stem cells (R1) generates the F[tk-neo] allele.

(B) Transient Cre recombinase expression and ganciclovir selection will result in ES subclones bearing αM-II$^{\Delta}$ (systemic-null) and αM-II^{F} (conditional-null) alleles.

(C) Genomic Southern blotting confirmed the expected αM-II allelic structures. Upper left: targeted ES clones 107 and 258 harbored the 7.5 kb (F[tk-neo]) allele and the 6.5 kb wild-type (wt) allele. Upper right: clone 107 bearing all three *loxP* sites was used in producing cells bearing αM-II^{F} (31–107) and αM-II$^{\Delta}$ (34–107) alleles. Lower panel: mice heterozygous and homozygous for the 6.5 kb αM-II$^{\Delta}$ allele are shown from Southern blot analyses of tail DNA. One-kilobase molecular weight ladders are in left lanes of above. Restriction enzyme sites Sma, SmaI; B, BamHI; K, KpnI; S, SacI; X, XbaI are denoted.

Complex N-Glycan Deficiency in Mutant Erythrocyte Membranes

A normal profile of membrane-associated and cytoskeletal proteins was observed in αM-II-deficient erythrocytes by SDS-PAGE and Coomassie blue staining, indicating that no changes occurred in the expression of the major membrane constituents (Figure 5A, left panel). Glycoproteins bearing complex N-linked oligosaccharides can be directly demonstrated by binding to the lectin E-PHA (Yamashita et al., 1983; Kobata and Endo, 1992). This binding requires the action of GlcNAc-TII, which cannot occur without αM-II (Figure 1). In wild-type erythrocytes, at least six glycoproteins bearing complex N-linked oligosaccharides were visualized by E-PHA binding (Figure 5A, right panel). This binding was absent from mutant samples, indicating that αM-II deficiency abrogates the production of complex N-glycans in erythrocytes. Mouse erythrocyte glycoproteins bearing complex N-linked oligosaccharides appeared upon close inspection to be distinct from those observed by Coomassie blue.

Erythroid Cells from αM-II-Null Mice Are Uniquely Deficient in Complex N-Glycans

Unexpectedly, analyses of various hematopoietic cells with E-PHA-biotin revealed that only erythocytes were deficient in complex N-glycans (Figure 5B). Thymocytes, myeloid cells, and mature lymphocytes displayed essentially normal levels of E-PHA binding, while erythroblast cell surfaces in the bone marrow were variably deficient. O-glycosylation appeared unaffected, as expected (Figure 5C); however, E-PHA histochemistry of the liver, kidney, spleen, pancreas, epidermis, and brain also indicated a normal level of cell surface complex N-glycans on αM-II-null cells (data not shown). Surprisingly, congenital loss of αM-II function appeared to ablate complex N-glycan production specifically in the erythroid lineage. This implied that an undisclosed alternate biosynthetic pathway was operating in other cell types. The nature of this alternate pathway was investigated by analyzing N-linked oligosaccharide biosynthesis and structural diversity in αM-II-deficient cells that retained E-PHA binding.

Structural Analyses of N-Glycan Biosynthesis in the Absence of αM-II

Splenocytes and primary fibroblasts were metabolically labeled with glucosamine or mannose for various times prior to isolating N-linked oligosaccharides for structural studies, using chromatography and exoglycosidase digestions. Complex N-glycan production in the absence of αM-II could occur from an altered lipid-linked oligosaccharide precursor lacking four mannose residues normally added by Dol-P-Man (Stoll et al., 1982). However, HPLC analyses did not reveal such an altered precursor in αM-II-deficient cells (Figure 6A). Newly synthesized oligosaccharide precursors are sometimes rapidly cleaved intact from lipid and protein; however, the proportion and size of those liberated during the labeling were also the same in control and mutant cells (data not shown). These results indicate that αM-II-null cells synthesize a normal lipid-linked oligosaccharide precursor and transfer this to protein as efficiently as wild-type cells.

ConA affinity chromatography is useful in determining the proportion of tetra-, tri- and biantennary complex, hybrid, and high mannose N-linked oligosaccharides (Baenziger and Fiete, 1979; Narasimhan et al., 1979). Surprisingly, αM-II-deficient cells contained N-linked oligosaccharides that eluted with the ConA-Sepharose