THE ROLE OF GOLGI α-MANNOSIDASE IIX IN GLYCAN PROCESSING

by

HARMINDER SINGH

(Under the Direction of Kelley Moremen)

ABSTRACT

N-linked glycoproteins are important in diverse cellular processes in mammals including development, self/non-self recognition and inflammation. At a molecular level, involvement of N-glycoproteins in these processes is seen as N-glycans regulating protein folding, targeting, recognition and adhesion processes. Studies employing a wide array of approaches such as animal models and mammalian cell lines have revealed that the class II mannosidases (CAZy family GH38), Golgi α-mannosidase II and Golgi α-mannosidase IIx, play an essential role in the conversion of hybrid-type N-glycans to complex-type N-glycans. Golgi α-mannosidase IIx is expressed as multiple transcript splice variants, only a subset of which encode functionally active enzymes in cell culture. The functional role for each of the splice variants is still unclear. We have expressed a recombinant, soluble form of Golgi α-mannosidase IIx that can cleave the hybrid-type native oligosaccharide substrate, $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_3GlcNAc_2$-PA, is unable to cleave high-mannose native oligosaccharide substrates, is activated 2.5 fold by cobalt, and is not detectable in mouse liver and kidney extracts as determined by immunoblotting. These data suggest that Golgi α-

mannosidase II and Golgi α-mannosidase IIx catalyze functionally redundant pathways in the Golgi apparatus.

INDEX WORDS: Golgi α-mannosidase IIx, Cytosolic α-mannosidase, Alternative splicing, N-linked glycosylation, Golgi apparatus

THE ROLE OF GOLGI α-MANNOSIDASE IIX IN GLYCAN PROCESSING

by

HARMINDER SINGH

B.S., Wayne State University, 1994

M.A., Wayne State University, 2000

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

THE ROLE OF GOLGI α-MANNOSIDASE IIX IN GLYCAN PROCESSING

by

HARMINDER SINGH

Major Professor: Kelley Moremen

Committee: Michael Pierce
Maor Bar-Peled
Alan Przybyla
Harry Dailey

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2010

ACKNOWLEDGEMENTS

I would like to ackowledge my mentor, Kelley Moremen, for inspiring me to reach a higher academic standard in every facet of my professional career. I would also like to acknowledge the past and present members of the Moremen lab for instilling a love of science in all of us. I would also like to thank members of the Pierce lab for all of their assistance in helping me complete my dissertation.

I would alko like to thank my family and my wife, Geena, for being there through these difficult times.

TABLE OF CONTENTS

Page

LIST OF TABLES

PAGE

LIST OF FIGURES

Page

ABBREVIATIONS

Abbreviations used: wt, wild-type; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; EST, expressed sequence tag; I/D, insertion/deletion; IS, intervening sequence; His, histidine; HA, human influenza hemagglutinin; Hs, *Homo* sapiens*;* Mm, *Mus* musculus; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; Dm, *Drosophila melanogaster*; ERGI, endoplasmic reticulum glucosidase I; ERGII, endoplasmic reticulum glucosidase II; ERMI, endoplasmic reticulum α-mannosidase I; UGGT, UDP-glucose:glycoprotein glucosyltransferase; CytMan, cytosolic α-mannosidase; PNGase, peptide:N-glycosidase; GMI, Golgi α-mannosidase IA, IB and IC; GNTI, N-acetylglucosaminyltransferase I; GMII, Golgi α-mannosidase II; GMIIx, Golgi α-mannosidase IIx; rGMIIx, recombinant Golgi α-mannosidase IIx; GNTII, N-acetylglucosaminyltransferase II; GMIII, Golgi α-mannosidase III; LysMan, lysosomal α-mannosidase; FUT10, fucosyltransferase 10; NAM, neutral α-mannosidase; MGAT5B, N-acetylglucosaminyltransferase Vb; MGAT6, N-acetylglucosaminyltransferase VI; EDEM3, endoplasmic reticulum degradation enhancer, mannosidase alpha-like 3; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalacosamine; Xyl, xylose; Fuc, fucose; Glc, glucose; Man, mannose; CDG, congenital disorders of glycosylation; HEMPAS, hereditary erythroblastic multinuclearity with a positive acidified serum test; CAZy, carbohydrate-active enzymes database; GH, glycosylhydrolase; DIM, 1,4-dideoxy-1,4-imino-D-mannitol; DMJ,

deoxymannojirimycin; KIF, kifunensine; SW, swainsonine; Dm, *Drosophila melanogaster*; pNP α-Man, p-nitrophenyl α-D-mannoside; 4-MU α-Man, 4-methylumbelliferyl α-D-mannoside; GSA II, *Griffornia simplicifolia* agglutinin II; E-PHA, erythroagglutinating phytohemagglutinin; MES, 2-(N-morpholino)ethanesulfonic acid); DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); BCA, bicinchoninic acid; CHT, ceramic hydroxyapatite; PBS, phosphate buffered saline; cDNA, complementary DNA; PA, pyridylamine; CHO, chinese hamster ovary; HEK, human embryonic kidney; MCF, Michigan Cancer Foundation; qRT-PCR, quantitative real time reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Glycoproteins are important players in diverse cellular processes and make major contributions to mammalian physiology and pathology. Protein-linked glycans can be divided into two major classes based upon the nature of the covalent bond. N-linked glycoproteins are modified with a carbohydrate moiety attached via an amide linkage to a consensus motif, N-(X)-S/T, where X is any amino acid except proline (Kornfeld and Kornfeld, 1985). O-linked glycoproteins in mammalian organisms differ from N-linked glycoproteins by the nature of the covalent bond where N-acetylgalactosamine (GalNAc), xylose (Xyl), fucose (Fuc), glucose (Glc), mannose (Man) or N-acetylglucosamine (GlcNAc) can be attached to the hydroxyl group of serine or threonine (Varki, et al., 2009). O-linked glycosylation has been extensively studied in a variety of mammlian biological contexts and will not be discussed as a part of the present work.

N-linked glycans play diverse roles in biological systems. Intracellularly, N-glycans contribute to the folding of initial translation products in the endoplasmic

reticulum (ER) and mediate the transport and function of mature glycoproteins (Helenius and Aebi, 2004). Extracellularly, N-glycans play important roles in cell adhesion events and recognition processes such as metazoan development, inflammatory responses, immune surveillance, hormone action and cell migration (Moremen, 2002, Helenius and Aebi, 2004). Alterations in cellular glycosylation events are currently being investigated as disease markers based on evidence that such changes have been shown to contribute to cell migration and metastasis in cancer, immune disorders, mental retardation, anemia and development (Moremen, 2002, Zhao, et al., 2008).

THE N-LINKED GLYCOSYLATION BIOSYNTHETIC PATHWAY

The biosynthesis of N-linked glycoproteins is initiated in the ER by the synthesis of a lipid linked oligosaccharide (Fig. 1.1) (Helenius and Aebi, 2004). Two N-acetylglucosamine (GlcNAc) and five mannose (Man) residues are added to dolichol phosphate on the cytosolic face of the ER where it is subsequently flipped to the lumenal face (Kornfeld and Kornfeld, 1985). The remaining four mannose and three glucose (Glu) residues are added and the oligosaccharide is transferred *en bloc* to a newly synthesized protein by oligosaccharyltransferase (OST) at the consensus motif N-(X)-S/T (Kornfeld and Kornfeld, 1985). This step is not absolute, as only two thirds of these sequons have been found to be modified on mature N-linked glycoproteins, contributing to the heterogeneity of N-linked glycosylation in eukaryotic organisms (Varki, et al., 2009).

N-glycan biosynthesis continues in the endoplasmic reticulum with the trimming of three glucose residues that are removed by ER α-glucosidase I (ERGI) and ER α-glucosidase II (ERGII) followed by trimming with ER α-mannosidase I (ERMI) to produce a $Man_8GlcNAc_2$ structure from the original $Glc_3Man_9GlcNAc_2$ structure (Fig. 1.2) (Weng and Spiro, 1996, Moremen, 2000, Moremen, 2002). One of the major contributions of the N-linked glycosylation biosynthetic pathway is to an ER quality control system that insures that proteins are properly folded prior to maturation. Here, the initial process of glucose trimming is followed by the selection of improperly folded glycoproteins by the calnexin-calreticulin cycle. Glycoproteins that are incompletely folded are trimmed to a $GlcMan_9GlcNac_2$ structure and are recognized by the lectin function of calnexin and calreticulin (Helenius and Aebi, 2004). The glucose residue can be cleaved by ERGII to generate a $Man_9GlcNac_2$ structure on the N-glycoprotein rendering it capable for further processing by ERMI and export to the secretory pathway (Moremen, 2002, Vembar and Brodsky, 2008). Alternatively, the N-glycoprotein can re-enter the calnexin-calreticulin cycle following reglucosylation of the N-glycan by UDP-glucose:glycoprotein glucosyltransferase (UGGT), the enzyme that acts as a folding sensor (Helenius and Aebi, 2004, Vembar and Brodsky, 2008). This iterative cycle can continue until the protein is folded or until the improperly folded glycoproteins are targeted for degradation by the proteosome in a process known as endoplasmic reticulum associated degradation pathway (ERAD) (Lippincott-Schwartz, et al., 1988, Vembar and Brodsky, 2008). It is believed that ERMI acts as the timing mechanism in selecting incorrectly folded glycoproteins for degradation (Helenius and Aebi, 2004).

Glycans derived from incorrectly folded N-glycans can be released and processed by a catabolic pathway compartmentalized in the cytosol and lysosomes. Free oligosaccharides are released from the incorrectly folded protein by peptide:N-glycosidase (PNGase) and subsequently trimmed to $Man_5GlcNac_2$ structures by the cytosolic α-mannosidase (CytMan) (Spiro, 2004). The free oligosaccharides are subsequently transported to the lysosome where the oligosaccharide is completely processed (Spiro, 2004).

Within the secretory pathway, correctly folded proteins are further processed in the Golgi complex to generate three types of N-glycan structures: high mannose-type, hybrid-type and complex-type oligosaccharides (Kornfeld and Kornfeld, 1985). The three types of oligosaccharides share a common core structure, $Man_3GlcNAc_2$ (Kornfeld and Kornfeld, 1985). The high mannose-type N-glycans have two to six additional mannose residues attached to the core structure, hybrid-type N-glycans have features of both high-mannose and complex-type oligosaccharides, and complex-type oligosaccharides are modified with additional non-mannose residues (Kornfeld and Kornfeld, 1985).

The diversification of N-glycans in the Golgi apparatus occurs in a species-dependent manner. In fungal organisms, such as *Saccharomyces cerevisiae*, the high mannose structure is elaborated with the addition of numerous mannose residues leading to hypermannosylated N-glycans with more than 100 mannose residues (Hamilton, et al., 2003). In metazoan organisms, the high mannose oligosaccharide structure is processed to complex type oligosaccharides by Golgi resident glycosidases and glycosyltransferases (Moremen, 2002). Processing is initiated by ERMI, which

cleaves $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ followed by a collection of Golgi α-mannosidase I (GMI) enzyme isoforms, (GMIA, GMIB and GMIC), that processes $Man_8GlcNAc_2$ to the key intermediate, $Man_5GlcNAc_2$ (Moremen, 2002).

Processing of high mannose oligosaccharides to hybrid type structures occurs through the combined action of GMI and N-acetylglucosaminyltransferase I (GNTI). GNTI initiates the synthesis of hybrid type oligosaccharides in the *cis*- to *medial*- Golgi by adding an N-acetylglucosamine residue to the Man α1→3 Man β1→ terminus of $Man_5GlcNAc_2$, forming the hybrid type oligosaccharide, $GlcNAcMan_5GlcNAc_2$.

Hybrid type oligosaccharides are converted to complex type oligosaccharide structures by the sequential action of Golgi α-mannosidase II (GMII), and possibly an alternative isoform homolog Golgi α-mannosidase IIx (GMIIx), by removing the terminal α 1,3 and α 1,6 mannose residues on $GlcNAcMan_5GlcNAc_2$, generating a $GlcNAcMan_3GlcNAc_2$ core structure, representing the key step in the conversion to complex type oligosaccharides (Tulsiani, et al., 1982, Moremen, 2002, Akama, et al., 2006, Shah, et al., 2008). N-acetylglucosaminyl transferase II (GNTII) completes the process by adding a β 1,2 GlcNAc onto the core terminal α 1,6 mannose branch, generating a complex-type oligosaccharide (Tan, et al., 1995).

It has also been suggested that high mannose type oligosaccharides could be converted into complex type oligosaccharides by an alternative processing pathway bypassing GMII and GMIIx based on glycan structural and enzymatic studies with the GMII null mouse (Chui, et al., 1997). According to the model, high mannose oligosaccharides could be converted from $Man_5GlcNAc_2$ to $Man_3GlcNAc_2$ by a Golgi α-mannosidase activity followed by conversion to complex type oligosaccharides by the

sequential action of GNTI and GNTII (Chui, et al., 1997). The existence of the alternate bypass pathway *in vivo* now appears to be unlikely due to recent studies demonstratingthe absence of complex type glycosylation and an embryonic lethal phenotype in the GMII/GMIIx double null mouse as discussed further below (Akama, et al., 2006).

The core complex-type structure with multiple non-reducing GlcNAc termini can undergo elaboration by the addition of branches and branch elongation generating a great diversity of oligosaccharide structures among proteins, cell types, tissues and species (Helenius and Aebi, 2001). In mammalian organisms, the complex-type oligosaccharides generated by GNTII are elaborated through the action of up to 4 N-acetylglucosaminyltransferases and decorated with galactose, N-acetylgalactosamine, sialic acid, fucose, glucuronic acid and sulfate residues by Golgi glycosyltransferases and sulfotransferases (Varki, et al., 2009).

N-GLYCOSYLATION AND HUMAN DISEASE

The role of N-linked glycosylation in pathogenesis is becoming increasingly important with approximately 1% of the genes in the mammalian genome being responsible for carbohydrate biosynthesis and modification (Ohtsubo and Marth, 2006). In particular, the Golgi glycosidases and glycosyltransferases are promising targets of genetic and biochemical studies focused on gaining insights into pathogenesis.

N-glycans have been found to have diverse roles in numerous physiological processes, including development, reproduction, pulmonary physiology, endocrinology,

hematology, immunology and neurobiology (Varki, et al., 2009). On a molecular level, N-glycans are important in cellular processes such as protein folding, adhesion, self/ nonself immune recognition, trafficking, receptor activation and endocytosis, cell-cell communication, and localization (Ohtsubo and Marth, 2006, Moremen, 2000). The diversity of proteins and processes affected by N-linked glycosylation provides an approach for studying the role of N-glycans in development and disease (Moremen, 2002).

In addition to employing *in vitro* approaches to study N-linked glycosylation, *in vivo* approaches utilizing human and animal models of disease have generated invaluable insights into the nature of the N-glycan biosynthetic pathway (Ioffe and Stanley, 1994, Moremen, 2002). One such disorder is congenital dyserythropoietic anemia type II or hereditary erythroblastic multinuclearity with a positive acidified serum test (HEMPAS). Although prevalence data is unavailable, 303 cases have been identified corresponding to a frequency of $<5.0 \times 10^{-6}$ % in the human population (Heimpel, et al., 2003). The disease is characterized by ineffective erythropoiesis, bone marrow erythroid multinuclearity, erythroid membrane abnormalities and secondary tissue siderosis (Fukuda, 1990, Moremen, 2002). Patients develop mild to severe anemia in combination with hepatosplenomegaly and reports of jaundice, gallstones and diabetes (Fukuda, 1990, Fukuda, 1993, Fukuda, 1999, Moremen 2002). On a molecular level, this is seen as a reduction in polylactosamine structures on the erythrocyte membrane glycoproteins, bands 3 and 4.5 (Fukuda, et al., 1994, Moremen 2002). Patients who develop the disease have been shown to have loss-of-function defects in the GMII coding region, but other studies have implicated altered expression

of N-acetylglucosaminyltransferase II or β 1,4 galactosyltransferase (Fukuda, et al., 1990, Fukuda, 1999, Moremen 2002). These data suggest that the disorder may not be exclusively a result of an enzymatic defect, but, rather, altered transcription or trafficking may also induce the occurrence of the disease (Moremen, 2002).

Alternatively, defects in Golgi N-glycosylation have been detected in type II congenital disorders of glycosylation (CDG). The first human deficiency in Golgi glycosylation was identified by the structural analysis of serum transferrin oligosaccharides and was termed CDG IIa (Jaeken, et al., 1994). CDG IIa patients have extremely reduced N-acetylglucosaminyltransferase II activity and severe psychomotor retardation (Remaekers, et al., 1991, Jaeken, et al., 1993). Presently, there are 22 known types of CDGs divided into two classes; CDG type I are defects in the generation of the N-glycan lipid-linked precursor whereas CDG type II are characterized by defects in glycan processing following transfer of the glycan precursor to the polypeptide backbone (Freeze, 2007). One of these human diseases, CDG Ib, is currently treatable (Marquadt and Denecke, 2003). Since the structural changes in N-linked glycans cannot be easily studied in human patients, mouse models of the biosynthetic enzymes have been produced.

The gene encoding N-acetylglucosaminyltransferase I was disrupted in the mouse and it was found that GNTI null mice die at embryonic days 9-10 with severe developmental abnormalities in neural tube formation and vascularization (Ioffe and Stanley, 1994, Metzler, et al., 1994). These studies have also shown that GNTI is essential for the conversion of hybrid-type oligosaccharides to complex-type oligosaccharides (Ioffe and Stanley, 1994). Similarly, the gene encoding N-

acetylglucsoaminyltransferase II was disrupted in the mouse and it was shown that mice lacking the *Mgat2* gene die in early postnatal development with severe gastrointestinal, hematologic and osteogenic abnormalities (Wang, et al., 2001). Novel N-glycan branched structures not found in the wild-type mouse were also detected in the *Mgat2* null mice (Wang, et al., 2001). Since the phenotype of the null mouse matches symptoms displayed by human patients with congenital disorder of glycosylation type IIa (CDG IIa), these data suggest that altered glycosylation leads to the incidence of human disease and, thus, provides an opportunity to develop therapeutic approaches (Wang, et al., 2001).

In conjuction with the gene disruptions in the glycosyltransferases GNTI and GNTII, a genetic disruption of Golgi α-mannosidase II in the mouse was generated (Chui, et al., 1997). The mice developed a dyserythropoietic anemia similar to HEMPAS, splenomegaly and immature erythrocytes or reticulocytes in the peripheral blood (Chui, et al., 1997). However, in contrast to HEMPAS, no hepatomegaly, liver cirrhosis or hemosiderosis was detected (Chui, et al., 1997, Moremen, 2002). Structural analysis of N-glycans from the null mouse revealed that expression of high mannose-type oligosaccharides are unaffected, however, in cells from the erythroid lineage, expression of hybrid-type oligosaccharides are elevated 3-fold and expression of complex-type oligosaccharides is reduced 50% (Chui, et al., 1997, Moremen, 2002). Biochemical studies on the substrate specificity of α-mannosidase activities in the null mouse tissues found no activity against $GlcNAcMan_5GlcNAc_2$, however, a novel activity for cleavage of $Man_5GlcNAc$ in the presence of Co^{2+} was detected and termed Golgi α-mannosidase III (GMIII) (Chui, et al., 1997, Moremen, 2002). Collectively, the data suggested that

another mannosidase was responsible for the production of complex type oligosaccharides in most tissues in the GMII null mouse and an alternative high mannose processing pathway exists in the Golgi that could bypass the GMII defect. A candidate enzyme for catalyzing the alternate pathway was GMIIx based on amino acid sequence homology to GMII and initial characterization of substrate specificity. The role of the enzyme in the conversion of hybrid N-glycans to complex N-glycans is the focus of this dissertation.

Identifying the enzymes responsible for the generation of complex type N-glycans is important from the standpoint that N-glycans are known to play key roles in cell-cell adhesion events, however, the molecular basis of these interactions remains unclear (Akama, et al., 2002, Ohtsubo and Marth, 2006). Since it was possible that GMIIx was involved in the conversion of hybrid type oligosaccharides to complex type oligosaccharides in the GMII null mouse, a GMIIx null mouse was subsequently generated and analyzed. Surprisingly, cells from the erythroid lineage were unaffected, however, mutant males were infertile (Akama, et al., 2002). Histological and biochemical analysis of the testes revealed that the mice had impaired spermatogenesis due to a failure of Sertoli cell-germ cell adhesion (Akama, et al., 2002). Furthermore, structural analysis of the N-glycans expressed in the testes showed that a previously unidentified GlcNAc-terminated tri-antennary and fucosylated oligosaccharide plays a key role in normal spermatogenesis (Fig. 1.3) (Akama, et al., 2002).

In order to address the question of whether GMIIx was catalyzing an alternative pathway in the conversion of hybrid type to complex type N-glycans, a GMII/GMIIx double null mouse was generated (Chui, et al., 1997, Akama, et al., 2006). The double

null mice died shortly after birth due to respiratory failure and subsequent histological analysis of the mice showed that the lungs had smaller alveoli and the lungs and liver had abnormal vacuole formation and enlarged mitochondria (Akama, et al., 2006). Lectin binding experiments showed that expression of high mannose type N-glycans were unaffected, however, expression of complex type N-glycans in the double null mouse embryo was not detected (Akama, et al., 2006). N-glycans expressed in mouse embryos followed three patterns: wild-type and GMIIx null mouse embryos expressed mostly complex-type N-glycan structures, GMII null mouse embryos expressed mainly hybrid-type N-glycan structures, and GMII/GMIIx double null mouse embryos did not express complex-type N-glycans at all (Akama, et al. 2006). Importantly, it was shown that complex-type N-glycan expression can be restored by transfecting double null fibroblasts with either a mouse GMIIx or mouse GMII cDNA (Akama, et al., 2006). These studies also demonstrated that recombinant, soluble mouse GMIIx hydrolyzes $GlcNAcMan_5GlcNAc_2$-PA to produce $GlcNAcMan_3GlcNAc_2$-PA, but the enzyme is unable to process $Man_6GlcNAc_2$-PA or $Man_5GlcNAc_2$-PA (Akama, et al., 2006). Taken together, the data revealed that mouse GMIIx appears to catalyze the functionally redundant pathway identified in the GMII null mouse (Akama, et al., 2006). More detailed structural analysis of N-glycans expressed in the GMII/GMIIx double null embryo revealed that GMIIx is responsible for the production of ~20% of complex-type N-glycans in GMII null mouse embryos, and four unusual hybrid-type oligosaccharides and two novel pseudocomplex-type oligosaccharides are produced (Fig. 1.4) (Hato, et al., 2006). These data provide evidence that altered glycan expression leads to physiological and developmental abnormalities coincident with pathological conditions

CLASSIFICATION OF ALPHA-MANNOSIDASES

α-Mannosidases are divided into three classes based on their structural, functional and biochemical properties (Henrissat, 1991, Daniel, et al., 1994, Moremen, et al., 1994, Moremen, 2002). Class I (CAZy GH family 47) mannosidases are primarily responsible for early trimming events in the ER and the Golgi apparatus, cleaving $Man_9GlcNAc_2$ structures to $Man_5GlcNAc_2$ structures (Daniel, et al., 1994, Moremen, et al., 1994). Class II (CAZy GH family 38) mannosidases have multiple family members playing roles in both glycoprotein maturation in the Golgi complex and glycoprotein catabolism in lysosomes and the cytosol (Daniel, et al., 1994, Moremen, et al., 1994, Moremen, 2002). In animal systems a third family of α-mannosidases (CAZy GH family 99) contains two members, one of which has been characterized as an endo-α-mannosidase that cleaves $Glc_{3\text{-}1}Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ during glycoprotein maturation (Daniel, et al., 1994, Moremen, et al., 1994).

CLASS I MANNOSIDASES

Class I (GH 47) mannosidases are primarily found in the endoplasmic reticulum and the *cis*-Golgi where the enzymes are involved in glycoprotein biosynthesis, protein folding and quality control (Moremen, 2000, Helenius and Aebi, 2004). The members of this family are characterized by having a conserved 440- to 510- amino acid $(\alpha\alpha)_7$ barrel catalytic domain (Vallee, et al., 2000, Vallee, Lipari, et al., 2000, Romero, et al., 2000), specificity for cleaving α 1,2 mannose linkages, a requirement of Ca^{2+} for

catalytic activity, sensitivity to inhibition by the pyranose substrate mimics deoxymannojirimycin (DMJ) and kifunensine (KIF) (Daniel, et al., 1994), and cleaving glycosidic linkages by the inversion of configuration of the released mannose glycone residue (Lipari, et al., 1995, Lal, et al., 1998, Moremen, 2002).

CLASS II MANNOSIDASES

Class II (GH 38) α-mannosidases are a heterogenous collection of processing and catabolic enzymes present in the ER, Golgi apparatus, lysosomes and cytosol of mammalian cells with homologs in other species ranging from archea to insects (Moremen, 2002). Members of the family are characterized by being relatively large (110-135 kDa), having a possible requirement for divalent cations for catalytic activity, sensitivity to inhibition by the furanose transition state analogs swainsonine (SW) and 1,4-dideoxy-1,4-imino-D-mannitol (DIM) (Daniel, et al., 1994), and cleaving glycosidic linkages by retention of anomeric configuration of the released monosaccharide (Howard, et al., 1997, Moremen, 2002). In addition, all of the class II α-mannosidases contain a restricted region of conserved sequence corresponding to the catalytic domain of the *Drosophila melanogaster* Golgi α-mannosidase II structure (van den Elsen, et al., 2001, Moremen, 2002)

There are five principal class II mannosidases. The first of these is the broad specificity lysosomal mannosidase (LysMan) involved in glycoprotein catabolism. The purified enzyme has a pH optimum of 4.0-4.5 (Liao, et al., 1996, Tollersrud, et al., 1997, Berg, et al., 2001, Moremen, 2002). The second enzyme is a core specific α 1,6 lysosomal

α-mannosidase. It is thought that the enzyme works in functional collaboration with the broad specificity lysososmal mannosidase to efficiently process oligosaccharides in glycoprotein catabolism (Park, et al., 2006). The third enzyme in the family is a soluble cytosolic α-mannosidase involved in oligosaccharide catabolism of incorrectly folded glycoproteins destined for degradation (Moremen, 2002, Spiro, 2004). The enzyme has been purified, cloned and appears to prefer Co^{2+} for catalytic activity (Shoup and Touster, 1976, Bischoff, et al., 1990, Moremen, 2002).

The fourth member in the family is Golgi α-mannosidase II. GMII has been extensively studied where it has been shown that the enzyme catalyzes the committed step in complex-type N-glycan production (Chui, et al., 1997). GMII has a short (~5 aa) cytoplasmic tail, a single transmembrane domain, a luminal "stem" domain (~80 aa) and a large luminal catalytic domain (~1000 aa) (Moremen, 2002). The structure of GMII was determined by X-ray crystallography of a recombinant Dm GMII protein (van den Elsen, et al., 2001). During the cloning of GMII, the fifth member of the family was discovered, Golgi α-mannosidase IIx (GMIIx) (Misago, et al., 1995).

In addition to the aforementioned members of the family, other α-mannosidases have been characterized that share biochemical properties with class II enzymes. One enzyme has been purified from rat liver microsomes and is inhibited by swainsonine, stimulated by Co^{2+}, and cleaves $Man_{9-4}GlcNAc_2$ to produce $Man_3GlcNAc_2$ (Bonay and Hughes, 1991, Moremen, 2002). Another enzyme has been identified in rat brain microsomes and, similar to the enzyme identified by Bonay and Hughes, cleaves $Man_{9-4}GlcNAc_2$ to produce $Man_3GlcNAc_2$ (Tulsiani and Touster, 1985). The genes corresponding to these latter activities have not yet been identified (Moremen, 2002)

GOLGI ALPHA-MANNOSIDASE CATALYTIC MECHANISMS

Along with the method of classifying α-mannosidases into broad families, structural approaches provide valuable information about the active site and catalytic mechanism. This information is important for the design of therapeutic compounds.

Golgi α-mannosidases hydrolyze a glycosidic bond via general acid catalysis requiring a proton donor and a nucleophile/base (Koshland, 1953, Sinnott, 1990, Davies and Henrissat, 1995). Hydrolysis occurs via two mechanisms giving rise to either an overall retention or inversion of anomeric configuration (Koshland, 1953, Davies and Henrissat, 1995).

INVERTING MECHANISMS

During catalysis with an inverting mechanism, the events occur simultaneously in one step (Davies and Henrissat, 1995). The glycosidic oxygen is protonated, the aglycon departs, and a water molecule activated by the nucleophilic base attacks the anomeric carbon (Davies and Henrissat, 1995). This mechanism leads to a product with an inverted stereochemistry as compared to the substrate (Davies and Henrissat, 1995). Class I Golgi α-mannosidases are classified by their biochemical properties, including the fact that the enzymes share an inverting mechanism for catalysis. The best characterized model for class I α-mannosidases is ERMI. The crystal structure of the protein has been solved in the absence or presence of the inhibitors kifunensine and 1-deoxymannojirimycin (Vallee, et al., 2000). The structure shows a role for the Ca^{2+},

where it coordinates the O-2' an O-3' hydroxyls of the mannose glycone and stabilizes the bound inhibitors in a $^1C_4$ conformation (Vallee, et al., 2000). The Ca^{2+} is also involved in coordination of the water nucleophile in the inverting mechanism of action.

RETAINING MECHANISMS

In contrast to the inverting mechanism, catalysis via a retaining mechanism occurs in two-steps (Davies and Henrissat, 1995). In the first step, the glycosidic oxygen is protonated by an acid catalyst and an enzyme-associated nucleophile assists in aglycone departure leading to the formation of a covalent intermediate with an inverted configuration(Davies and Henrissat, 1995). In the second step, the glycosyl-enzyme intermediate is hydrolyzed by a water molecule generating a product with an inverted configuration relative to the covalent intermediate, but a retained configuration relative to the original substrate (Davies and Henrissat, 1995). Similar to class I α-mannosidases, class II α-mannosidases have been studied through work on a model enzyme, GMII. The crystal structure of Dm GMII has been solved in the presence and absence of the inhibitors swainsonine and deoxymannojirimycin (van den Elsen, et al., 2001). It has studied extensively as a model for substrate binding and catalysis (van den Elsen, et al., 2001, Shah, et al., 2008). In the latter studies, an enzyme associated Zn^{2+} ion contributes to substrate binding and the retaining mechanism is catalyzed by the nucleophilic attack by D204 and an acid/base function of D341.

Although much information has been gathered as a result of structural analysis of Dm GMII, interpretation of data from functional approaches on related enzymes from other sources is complicated. For example, Golgi α-mannosidase IIx is classified as a GH38 α-mannosidase based on evidence gathered through genetic and biochemical approaches. However, the functional role of GMIIx is still poorly understood, particularly for the human enzyme. This enzyme is believed to catalyze a functionally redundant pathway with GMII based on mouse knockouts and biochemical characteristics of the murine enzyme (Akama, et al., 2006), but discrepancies in reported substrate specificities for the human enzyme renders it unclear which biochemical pathways are catalyzed by GMIIx (Oh-Eda, et al., 2001, Akama, et al., 2006). Similarly, although essential roles for GMIIx have been found in spermatogenesis and pulmonary function (Akama, et al., 2002, Akama, et al., 2006), there remains a need to characterize the function of the enzyme more extensively on a molecular level.

Studies on N-glycan biosynthesis in mutant cell lines have also contributed to our understanding of N-linked glycosylation biochemical pathways by providing tools to study functions in addition to human and animal models. Notable are the Lec mutants that are blocked in the formation of complex-type oligosaccharides (Stanley, 1984). A recent report described the generation of a HEK293T cell line, Lec36, with a mutation in the *Man2a1* locus that encodes the enzyme Golgi α-mannosidase II (Crispin, et al., 2009). Interestingly, characterization of the cell line showed that the N-glycans

expressed on a reporter protein, s19A, were mostly hybrid-type oligosaccharides mimicking the phenotype seen in HEMPAS patients. These data indicate that Golgi α-mannosidase IIx does not significantly contribute to the production of complex-type N-glycans in this cell line possibly due to low transcript expression (Crispin, et al., 2009). This is an example of a tissue other than erythroid cells where GMIIx is unable to compensate for the loss of GMII activity.

REGULATION OF GLYCOSYLATION PATHWAYS

Cellular mechanisms that may regulate N-glycan expression include gene transcription, protein localization, protein degradation, competition for substrates and substrate availability (Ohtsubo and Marth, 2006, Nairn, et al., 2008). During the initial cloning of GMIIx, the cDNA cloned from a human melanoma cDNA library contained a frameshift leading to a premature stop codon at bp 2389 (Misago, et al., 1995). It has also been shown that other members of the N-linked glycosylation biosynthetic pathway are also alternatively spliced, including N-acetylglucsoaminyltransferase I, N-acetylglucosaminyltransferase II, glucosidase II, N-acetylglucosaminyltransferase VB and the α 1,3 fucosyltransferase FUT10 (Yang, et al., 1994, Arendt, et al., 1999, Kaneko, et al., 2003, Tsitilou and Grammenoudi, 2003, Mollicone, et al., 2009). Despite the presence of alternative transcript splicing for the above glycan processing genes, the potential role of regulated splicing in influencing glycan expression has not been established. One model being proposed for alterations in glycan levels is regulation of overall transcript levels for glycan related enzymes that leads to changes in glycan

expression. Numerous correlations were observed between glycan structures found in mouse tissues and corresponding transcript levels encoding enzymes involved in the elaboration of glycan structures (Nairn, et al., 2008). The importance of transcriptional regulation is seen by the induction of N-acetyglucosaminyltransferase V expression in response to proliferative signals from *her-2/neu* oncogene (Chen, et al., 1998, Ohtsubo and Marth, 2006).

GOLGI ALPHA-MANNOSIDASE IIX

Golgi α-mannosidase IIx is the protein translation product of the *Man2a2* gene. GMIIx was originally discovered during screening of a human genomic library for GMII when two clones were found that contained similar, but non identical nucleotide sequences compared to GMII (Misago, et al., 1995). Additional screening of a melanoma cDNA library resulted in the isolation of a cDNA that encodes an alternatively spliced transcript containing an additional 25 bp leading to a frameshift and premature termination (Misago, et al., 1995). Screening of lymphocyte poly(A)$^+$ RNA and the subsequent generation of a cDNA led to the isolation of a clone encoding a protein of 1139 aa with a 25 bp deletion when compared to the human melanocyte cDNA. The latter cDNA was shown to encode an enzyme with poor α-mannosidase activity against the synthetic substrate p-nitrophenyl α-D-mannoside (pNP α-Man) in transfected COS cells (Misago, et al., 1995). Northern blotting showed that GMIIx is expressed as a single transcript of 5.0 kb in human heart, brain, placenta, liver, skeletal muscle, kidney and pancreas tissues and the gene maps to chromosome 15q25 (Misago, et al., 1995).

The presence of multiple splice isoforms suggested that the alternative splicing of *Man2a2* transcripts may be important for the expression of full length, functional GMIIx. Furthermore, tissue-specific differences in GMIIx expression suggest that GMIIx may be transcriptionally regulated. It is now known that the GMIIx transcript contains 22 exons (Ensembl 2009). Primer-extension analysis of human melanoma poly $(A)^+$ RNA revealed the presence of six transcriptional start sites and analysis of the region 5' of the transcriptional start site showed that the region contains no classic TATA box, no CAAT box, and numerous transcription factor binding sites, including Sp1, γ-IRE, and MyoD (Ogawa, et al., 1996). Subsequent studies were performed to gain structural and functional data about its role in N-linked glycosylation. Immunohistochemistry of HeLa cells and COS cells transfected with GMIIx cDNA showed that GMIIx protein is localized to the Golgi apparatus (Fig. 1.5) (Oh-Eda, et al., 2001). The expressed enzyme was able to cleave the synthetic substrate, 4-methylumbelliferyl α-D-mannoside (4-MU α-Man), had a pH optimum of 5.5, was inhibited by swainsonine, and was unable to cleave natural oligosaccharide substrates in the alditol form (Oh-Eda, et al., 2001). Alongside the *in vitro* studies, *in vivo* studies of CHO cells transfected with GMIIx showed that the levels of $Man_6GlcNAc_2$ were reduced and levels of $Man_4GlcNAc_2$ were elevated (Oh-Eda, et al., 2001). Interestingly, no evidence was found for the shortage of the key intermediate, $Man_5GlcNAc_2$. This indirectly showed that GMIIx hydrolyzes $Man_6GlcNAc_2$ to $Man_4GlcNAc_2$, prompting the proposal of a model where GMIIx catalyzes an alternative biochemical pathway bypassing GMII (Fig. 1.6) (Oh-Eda, et al., 2001). A caveat of the prior studies was the

subsequent determination that the splice isoform employed was among the least active among those tested in the present studies.

To confirm the results in the mammalian cell line, a GMIIx null mouse was generated as described above, demonstrating defective spermatogenesis and impaired germ cell-Sertoli cell adhesion (Akama, et al., 2002). The identification of this glycan provides evidence for a role of Golgi α-mannosidase IIx in complex N-glycan formation and insight into the molecular basis of cell-cell adhesion in spermatogenesis.

THE CYTOSOLIC ALPHA-MANNOSIDASE

Characterization of the GMII null mouse suggested the presence of an alternative bypass pathway compensating for the absence of GMII in the synthesis of complex N-glycans (Chui, et al., 1997). It was found that an enzyme activity, termed "GMIII", was expressed in spleen, liver and kidney in the GMII null mice (Chui, et al., 1997). A possible candidate for this role is the cytosolic α-mannosidase based on similar biochemical properties to GMIII, as both enzymes have a pH optimum of 6.0-6.5 and are stimulated by Co^{2+}.

The cytosolic α-mannosidase, also known as the neutral α-mannosidase (NAM), is a Co^{2+} stimulated enzyme and was originally cloned from rat liver (Bischoff, et al., 1990, Costanzi, et al., 2006). The CytMan was also shown to be present in a processed form in an ER membrane fraction and termed ER mannosidase II (ERMII) although the latter enzyme was found to be a 82 kDa polypeptide in contrast to the 105 kDa cytosolic enzyme (Weng and Spiro, 1996). Biochemical characterization of the enzyme showed

that CytMan and ERMII are both resistant to inhibition by kifunenesine and sensitive to inhibition by DIM, unlike ERMI which is sensitive to kifunensine inhibition and resistant to DIM inhibition (Weng and Spiro, 1996). Assays with oligosaccharide substrates indicated that CytMan and ERMII cleave $Man_9GlcNAc$ to $Man_5GlcNAc$ via the C isoform of $Man_8GlcNAc$ and can cleave aryl glycoside synthetic substrates (Weng and Spiro, 1996). In contrast, ERMI cleaves $Man_9GlcNAc$ to from the B isoform of $Man_8GlcNAc$ and is unable to cleave aryl glycosides (Weng and Spiro, 1996). Immunoblotting of ERMII and CytMan with antibodies specific for the N- or C-terminus of CytMan showed that the antibody recognizing the C-terminus is immunologically cross reactive in the enzyme from both sources (Weng and Spiro, 1996). In contrast, the N-terminus of ERMII is not recognized by the anti-CytMan N-terminal antibody (Weng and Spiro, 1996). The difference in size between the 105 kDa CytMan and the 82 kDa ERMII suggests that CytMan is translocated into the ER where it can be proteolytically cleaved to form ERMII (Weng and Spiro, 1996). In support of the role of CytMan in catabolism of free oligosaccharides, it was shown that CytMan and ERMII are unable to process oligosaccharides bearing the di-N-acetylchitobiose moiety, instead, they only act on free oligosaccharides with a single GlcNAc at the reducing terminus (Oku and Hase, 1991, Weng and Spiro, 1996, Saint-Pol, et al., 1997).

The relevance of CytMan in pathogenesis is an active area of research. Down-regulation of *Man2C1* in HEK293 cells by siRNA treatment led to an increase of $Man_9GlcNAc$ and $Man_8GlcNAc$ in the cytosol suggesting a role for CytMan in the catabolism of free oligosaccharides (Suzuki, et al., 2006). Additionally, it is known that changes in cell motility and cellular proliferation lead to invasiveness. To investigate the

hypothesis that the maintenance of the cytoskeleton is regulated by the nature of the N-glycan structures on cytoskeletal proteins, changes in cellular behavior were examined following suppression of *Man2C1* by siRNA treatment. It was found that *Man2C1* suppresion led to the growth arrest of EC9706 human esophageal carcinoma cells in S and G_2-M phases and an inhibition of microtubule polymerization, suggesting a possible link between high mannose oligosaccharide expression and invasiveness (Tian, et al., 2008).

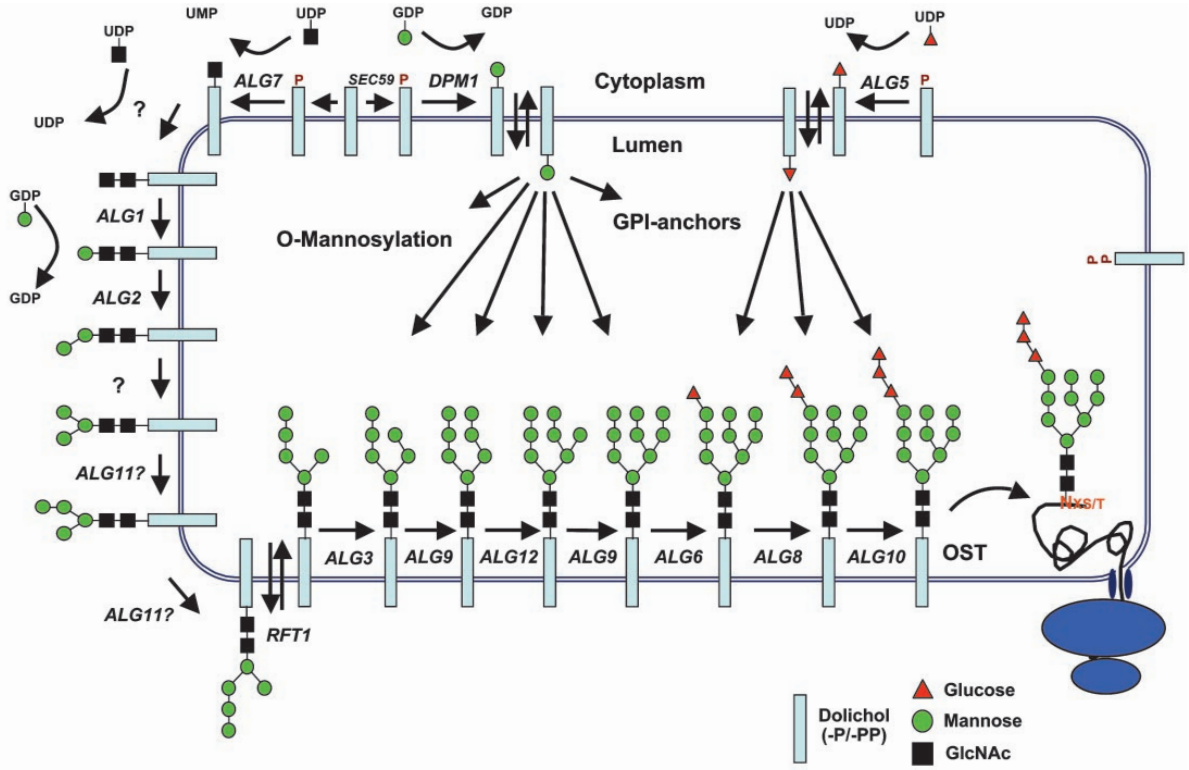


Fig. 1.1. N-glycoprotein biosynthesis is initiated in the endoplasmic reticulum. The yeast loci required for the individual steps are indicated. Two N-acetylglucosamine and five mannose residues are added to a dolichol phosphate precursor. Following the flipping of the oligosaccharide precursor from the cytosolic face to the lumenal face of the ER, four additional mannose residues and three glucose residues are added. The oligosaccharide moiety is subsequently transferred *en bloc* to newly synthesized proteins by oligosaccharyltransferase (OST).

Helenius, A. and M. Aebi. (2004). *Ann Rev Biochem* **73**:1019-1049.

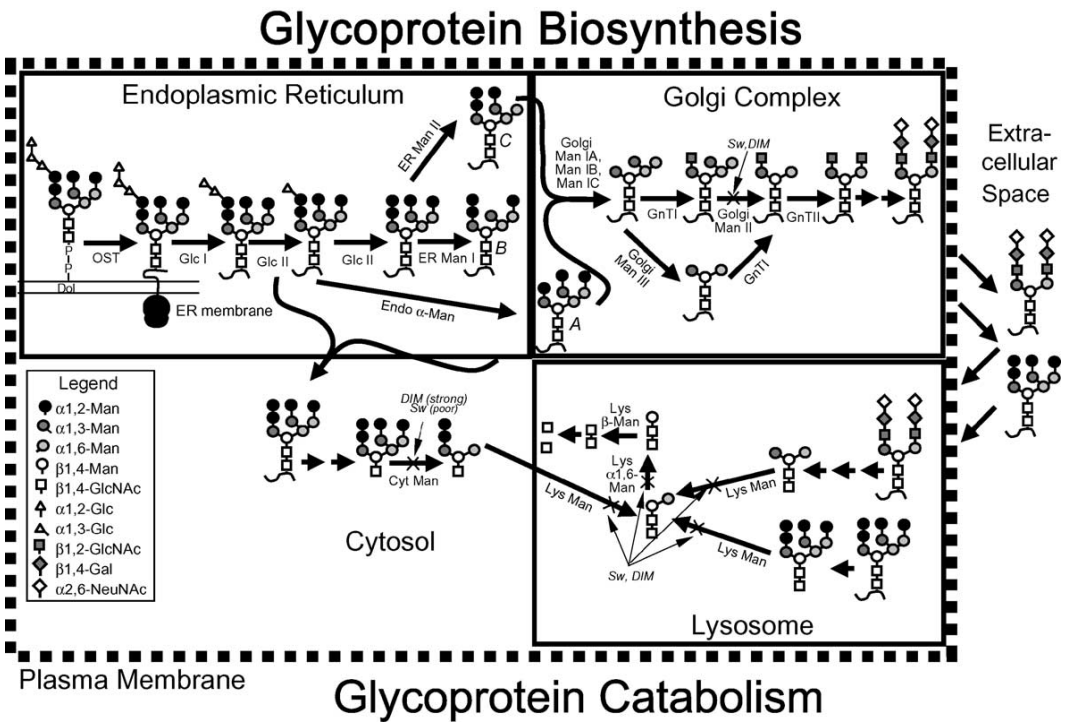


Fig. 1.2. The biochemical pathways of N-linked glycosylation are distributed among various organelles in metazoan cells where the biosynthetic pathways reside in the endoplasmic reticulum and the Golgi apparatus and the catabolic pathways reside in the cytosol and the lysosome. Abbreviations for enzyme activities are: Glc I, α-glucosidase I; Glc II, α-glucosidase II; ER Man I, ER mannosidase I; ER Man II, ER mannosidase II; Endo-α-Man, endo-α-mannosidase; Golgi Man IA, IB, IC, Golgi α-mannosidase IA, IB and IC; Golgi ManII, Golgi α-mannosidase II; Golgi ManIII, Golgi α-mannosidase III; GNTI, N-acetylglucosaminyltransferase I; GNTII, N-acetylglucosaminyltransferase II; Cyt Man, cytosolic α-mannosidase; Lys Man, lysosomal α-mannosidase; Lys α1,6Man, lysosomal α1,6 mannosidase; Lys β-Man, lysosomal β-mannosidase. Abbreviations for the inhibitory compounds are: Sw, swainsonine; DIM, 1,4-dideoxy-1,4-imino-D-mannitol.

Moremen, K. W. (2002). *Biochim Biophys Acta* **1573**(3):225-235.

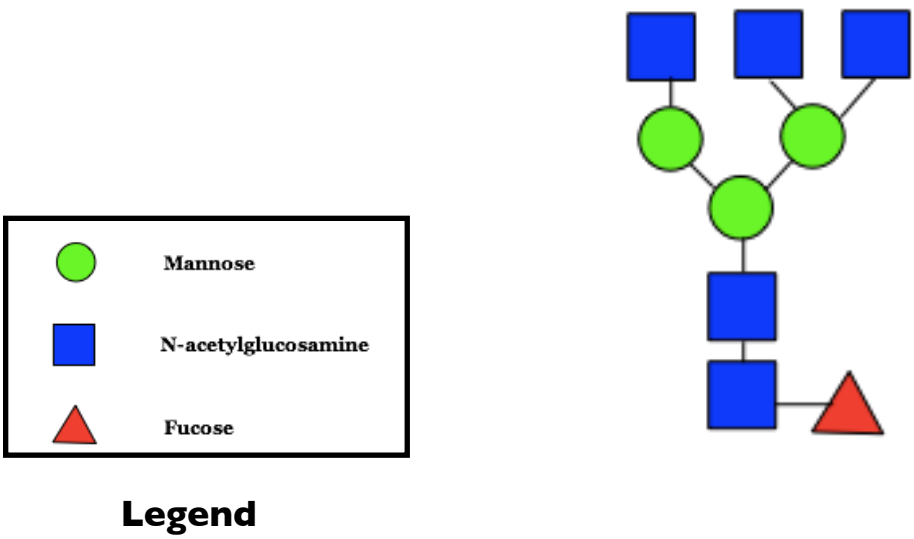


Fig. 1.3. The structure of an essential N-acetylglucosaminyl triantennary, fucosylated complex type oligosaccharide required for germ cell-Sertoli cell adhesion in the mouse during spermatogenesis. The sugar residues are depicted according to the standard established by the Consortium for Functional Glycomics.

Akama, et al. (2002). *Science* **295**(124):124-127.

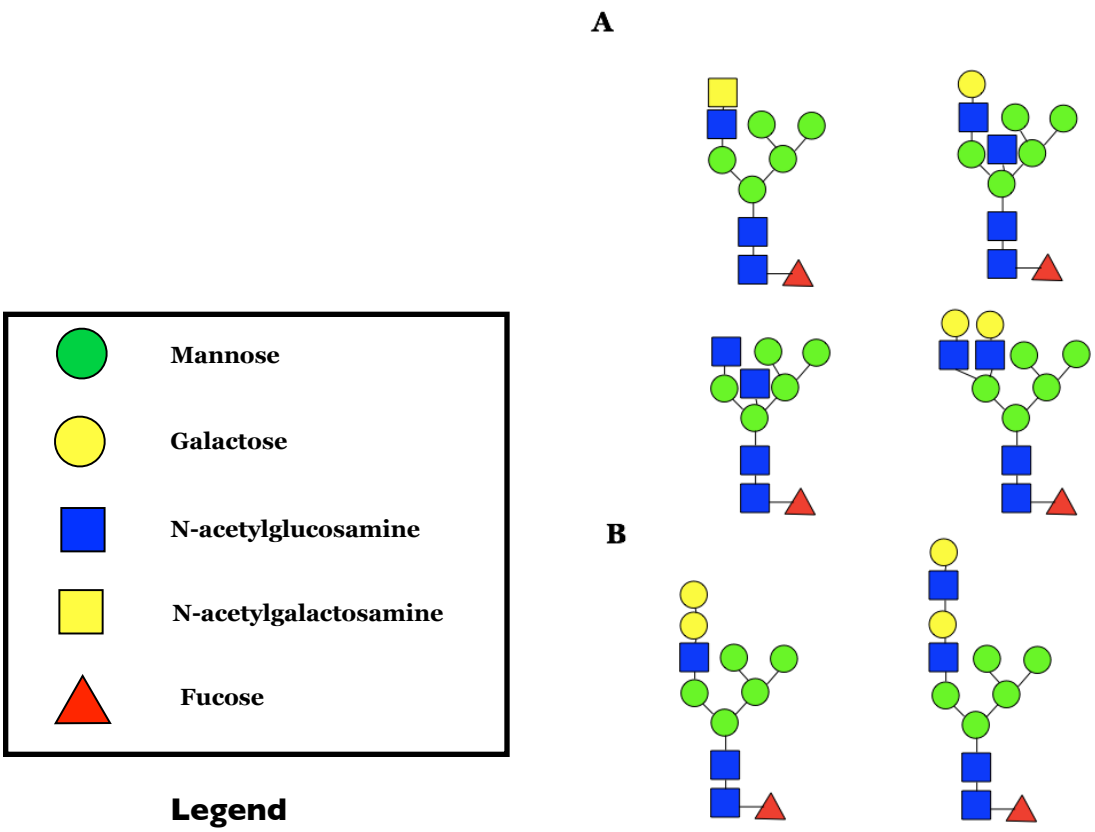


Fig. 1.4. Unusual and novel N-glycan structures identified in the GMII/GMIIx double null mouse. (A) The structures of four complex-type N-glycans containing N-acetylgalactosamine residues or additional branches uncharacteristic of N-glycans expressed in the wild-type mouse. (B) The structures of two novel pseudocomplex N-glycans. N-glycans were isolated from the GMII/GMIIx double null mouse using 2D LC mapping combined with MDSF-MALDI-TOF/TOF. The sugar residues are depicted according to the standard established by the Consortium for Functional Glycomics. Hato, et al. (2006). *Mol Cell Proteomics* **5**(11):2146-2157.

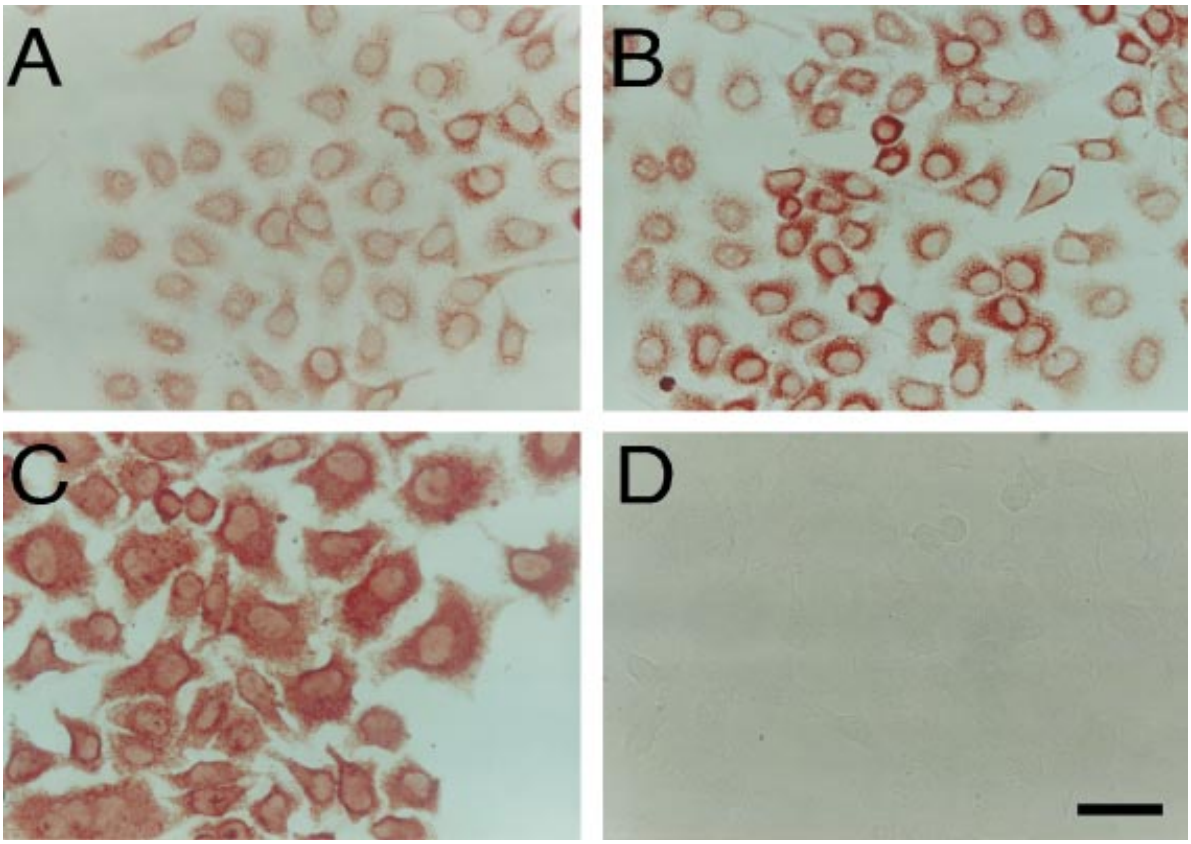


Fig. 1.5. Immunocytochemistry of HeLa cells with antibodies each for MX, MII and protein disulfide isomerase. The positive immunostainings are shown by red color deposit. (A) HeLa cells were stained with polyclonal anti-MX Ig followed by biotinylated second antibody and peroxidase-conjugated avidin. (B) HeLa cells were stained for MII (Golgi marker).(C) HeLa cells were stained for protein disulfide isomerase (ER marker). (D) Control, without the primary antibody. Scale bar = 25 mm.

Oh-Eda, et al. (2001). *Eur J Biochem* **268**:1280-1288.

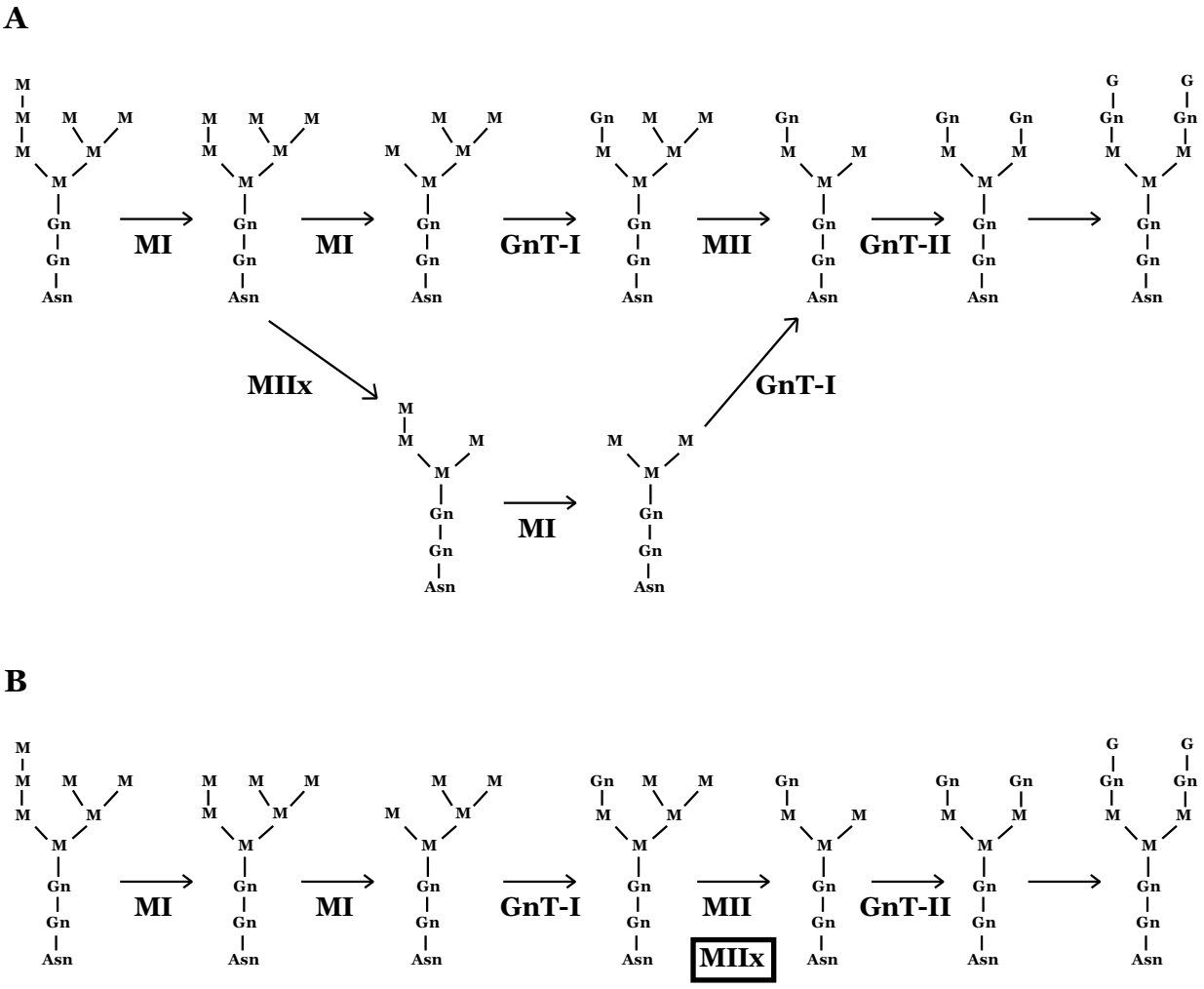


Fig. 1.6. Models of N-glycoprotein processsing in the Golgi apparatus. (A) A model where GMIIx catalyzes an alternative pathway to complex N-glycoprotein biosynthesis bypassing GMII. (B) An alternative model where GMII and GMIIx catalyze functionally redundant pathways. Abbreviations for enzyme activities are: MI, Golgi α-mannosidase I; GnT-I, N-acetylglucosaminyltransferase I; MII, Golgi α-mannosidase II; MIIx, Golgi α-mannosidase IIx; GnT-II, acetylglucosaminyltransferase II.

Oh-Eda, et al. (2001). *Eur J Biochem* **268**:1280-1288., Akama, et al. (2006). *Proc Natl Acad Sci U S A* **103**(24):8983-8988.

CHAPTER 2

GOLGI α-MANNOSIDASE IIx IS ESSENTIAL FOR COMPLEX N-GLYCAN FORMATION IN MAMMALS

ABSTRACT

The conversion of hybrid-type oligosaccharide structures to complex-type oligosaccharide structures in the Golgi apparatus is essential for metazoan development. Characterization of the biochemical steps involved in N-glycan processing employing animal models has demonstrated the existence of alternative pathways for the biosynthesis of complex-type N-glycans. The finding that complex-type N-glycans are still produced in the Golgi α-mannosidase II (GMII) null mouse led to the hypothesis of an uncharacterized GH38 α-mannosidase catalyzing the bypass pathway in the absence of GMII. Here, a homolog of GMII, Golgi α-mannosidase IIx (GMIIx), was investigated for its role in processing N-glycans in the Golgi apparatus. GMIIx is expressed as multiple splice isoforms in human muscle, spleen and testis tissues, only a subset of which encode functionally active enzymes in cell culture. A recombinant, soluble form of GMIIx was expressed in HEK293 cells. Purification and characterization of the enzyme showed that rGMIIx cleaves the hybrid-type native oligosaccharide $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_3GlcNAc_2$-PA, but is unable to process high

mannose-type native oligosaccharide substrates and is inhibited by swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol (DIM) similar to GMII. Unlike GMII, rGMIIx was stimulated 2.5 fold by cobalt. Based on the *in vitro* data, processing of hybrid-type oligosaccharide structures by GMIIx was examined in cell fractions prepared from mouse livers. Either in crude membrane preparations or following immunoprecipitation, no GMIIx activity was detected in any fractions, possibly due to interference from the anti GMIIx antibody or poor GMIIx protein expression in mouse livers. Additionally, the cytosolic α-mannosidase (CytMan) was investigated as a possible contributor to the "Golgi α-mannosidase III-like" (GMIII) activity identified in the GMII null mouse. The immunoprecipitation of an activity with the anti-CytMan antibody from mouse livers revealed a catalytic activity that cleaved the high-mannose native oligosaccharide substrate, $Man_5GlcNAc$-PA, in the presence of cobalt, suggesting that GMIII activity reflects a contamination of CytMan in the membrane preparations in the GMII null mouse. Collectively, these data indicate that human Golgi α-mannosidase IIx and human Golgi α-mannosidase II catalyze functionally redundant pathways in the conversion of hybrid-type N-glycans to complex-type N-glycans in the Golgi apparatus.

INTRODUCTION

Golgi α-mannosidase IIx plays an important biological role by mediating the conversion of hybrid type N-linked oligosaccharides to complex type oligosaccharides in the Golgi apparatus. Hybrid type N-glycans are processed in the Golgi by the sequential action of N-acetylglucosaminyltransferase I (GNTI), Golgi α-mannosidase II (GMII) or

its isozyme Golgi α-mannosidase IIx (GMIIx), and N-acetylglucosaminyltransferase II (GNTII) (Fig. 2.1) (Akama, et al., 2006). An alternate pathway has been proposed to bypass this route where high mannose N-glycans are processed by GMIIx or another mannosidase leading to the formation of the core oligosaccharide $Man_3GlcNAc_2$ that is converted to complex type oligosaccharides by the sequential action of GNTI and GNTII (Fig. 2.1) (Chui, et al., 1997, Oh-eda, et al., 2001). Although GMIIx remains a candidate enzyme for processing high mannose structures in the bypass pathway, it has not been shown *in vitro* that GMIIx is able to process the high mannose oligosaccharides, $Man_6GlcNAc_2$ or $Man_5GlcNAc_2$.

GMIIx was originially discovered while screening a human genomic library for GMII when two cDNAs were identified that had similar but not identical DNA sequences to GMII (Misago, et al., 1995). During characterization of the enzyme, it was found that GMIIx is transcribed as two splice variants, one containing a frameshift mutation leading to the production of a truncated nonfunctional protein of 796 amino acid residues and another with a 25 bp deletion leading to the synthesis of a polypeptide of 1139 amino acid residues (Misago, et al., 1995). Subsequent analysis showed that GMIIx is transcribed in most tissues as a 5 kb transcript, however, it remains unclear how GMIIx is transcriptionally regulated in a global manner (Misago, et al., 1995, Nairn, et al., 2008).

Based on sequence identity with GMII, it was assumed that GMIIx has similar biochemical characteristics and substrate specificity. Characterization of GMIIx revealed that the protein colocalizes to the Golgi apparatus with GMII in Hela cells, and does not colocalize with the endoplasmic reticulum (ER) marker, protein disulide isomerase

(PDI) (Oh-Eda, et al., 2001). Transfection of GMIIx into COS cells showed that the enzyme hydrolyzes the synthetic substrate 4-methyl-umbelliferyl-α-D-mannoside and has approximately 15% of the α-mannosidase activity of GMII (Oh-Eda, et al., 2001). However, when GMIIx was overexpressed in CHO cells, $Man_6GlcNAc_2$ levels become reduced and $Man_4GlcNAc_2$ levels were elevated implying that GMIIx is responsible for this conversion and the subsequent proposal of an alternative bypass pathway for processing of high mannose oligosaccharides catalyzed by GMIIx (Fig. 2.1) (Oh-eda, et al., 2001). To address the question of the exact substrate specificity of GMIIx, a recombinant, soluble catalytic domain of the murine variant of the enzyme was overexpressed in COS cells and it was found that mGMIIx hydrolyzes the pyridylamine (PA)-tagged oligosaccharide $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_3GlcNAc_2$,-PA but neither $Man_6GlcNAc_2$-PA nor $Man_5GlcNAc_2$-PA were cleaved (Akama, et al., 2006). It was also not reported if divalent cations, such as Co^{2+}, had any effect on GMIIx activity.

Similarly, functional studies of Golgi α-mannosidase IIx have generated important insights about its biological role in development. Analysis of the GMII null mouse showed that the mouse displays a dyserthryopoietic anemia similar to HEMPAS and non-erythroid tissues were able to synthesize complex type oligosaccharides at a reduced level (Chui, et al., 1997). Additionally, GH38 mannosidases were suspected to play a role in male reproductive development since farm animals ingesting locoweed, whose toxic agent, swainsonine, inhibits the GH 38 mannosidases, GMII and the broad specificity lysosomal mannosidase, develop male infertility (Akama, et al., 2002).

Based on these observations, a GMIIx null mouse with a germline disruption in *Man2a2* was generated. Analysis of the null mice showed that the males are infertile

with smaller testes and reduced number of spermatids in the testes and caudal ductyl epididymis (Akama, et al., 2002). Lectin histochemistry with *Griffornia simplicifolia* agglutinin II (GSA II) revealed that germ cells from GMIIx null mice express almost no GlcNAc terminated N-glycans (Akama, et al., 2002). Further biochemical characterization indicated that the defect in spermatogenesis was due to reduced expression of an essential GlcNAc-terminated tri-antennary fucosylated oligosaccharide leading to a failure of germ cell-Sertoli cell adhesion (Akama, et al., 2002).

To pursue the role of GMIIx in the production of complex type oligosaccharides, GMII/GMIIx double null mice with germline disruptions in both genes were generated and analyzed. The embryos died shortly after birth due to respiratory failure with abnormal vacuole formation and enlarged mitochondria in the lungs and liver (Akama, et al., 2006). Analysis of glycans from the embryos revealed that GMII/GMIIx double null mice do not express complex type oligosaccharides whereas GMIIx null embryos mirror the expression pattern of wild type embryos with mostly complex type glycans being expressed (Akama, et al., 2006). When GMIIx was transfected into GMII/GMIIx double null fibroblasts, complex type N-glycan expression was restored as determined by E-PHA lectin histochemistry (Akama, et al., 2006).

Collectively, these data suggest that GMIIx is expressed in a tissue-specific manner where the enzyme is responsible for the production of complex type N-glycans. The data also suggest GMIIx is expressed in tissues producing complex type oligosaccharides in the GMII null mouse. In this work, it is shown that human GMIIx and GMII catalyze functionally redundant pathways, confirming GMIIx as a major player in the biosynthesis of complex type N-glycans. Interestingly, the inability of

human GMIIx to hydrolyze high mannose oligosaccharides poses additional questions about its role in the alternative high mannose processing pathway.

RESULTS

Human Golgi α-Mannosidase IIx is Expressed as a Collection of Splice Isoforms

During the original cloning of the human GMIIx cDNA, it was found that the gene is transcribed as a collection of splice isoforms in a tissue specific manner, however, attempts to characterize the enzyme left the question of the substrate specificity unresolved (Misago, et al., 1995, Oh-Eda, et al., 2001). In order to determine the nature of GMIIx protein expression, the splice isoforms were compared at the protein sequence level. A protein sequence alignment of four GMIIx splice isoforms and human GMII revealed that the GMIIx isoforms and GMII display extensive regions of similarity, primarlily in the N-terminus of the protein containing residues important for catalysis based on studies with Dm GMII (Shah, et al., 2008). However, the four isoforms also show a region in the C-terminus of the proteins where the protein sequence diverges among the various transcripts (Fig. 2.2).

The region of protein sequence divergence in the C-terminus of GMIIx was examined in more detail among the splice isoforms. A GMIIx splice variant expressed in melanoma cells, GMIIx_v1b, is alternatively spliced to include an additional 25 bp of intronic sequence leading to a frameshift in the reading frame and the expression of a

stop codon (Fig. 2.3). This change in reading frame causes the expression of an internally truncated protein of 796 aa. The second splice variant, GMIIx_v2b, is expressed in human brain tissue. Alternative splicing leads to the transcription of a tissue specific exon and the translation of an additional 25 amino acids in the protein sequence (Fig. 2.4). The third splice variant, GMIIx_v3b, is expressed in melanoma cells. Alternative splicing leads to expression of alternative exon 15.1 and alternative exon 17. Transcription of alternative exon 15.1 leads to the expression of a base pair deletion and a frameshift in the protein sequence. Transcription of alternative exon 17 leads to the restoration of the reading frame in the protein sequence (Fig. 2.5).

Expression of the splice isoforms in human tissues was examined by RT-PCR. GMIIx_v1a versus GMIIx_v1b transcript expression was analyzed in human muscle, spleen and testis. In all of these tissues, the predominant transcript was GMIIx_v1a whereas GMIIx_v1b was not detected (Fig. 2.6). The same tissues were analyzed for expression of the GMIIx splice isoforms, GMIIx_v2a and GMIIx_v2b. In all of these tissues, the predominant transcript that was detected is GMIIx_v2a whereas GMIIx_v2b expression was detected in small amounts in human muscle and testis (Fig. 2.7). Collectively, these data suggest that the predominant splice isoforms expressed in human muscle, spleen and testis tissues are GMIIx_v1a and GMIIx_v2a leading to the translation of a putative full-length wild-type protein.

In conjuction with the studies in human tissues, GMIIx expression was examined in mouse livers. Transcript expression of various genes involved in N-glycan processing were quantitated by qRT-PCR (Fig. 2.8) (Nairn, et al., 2008). Transcript levels of GMII and GMIIx were similar to other N-glycan biosynthetic enzymes such as ERMI, GMI,

GNTI and GNTII indicating that the GMIIx transcript was being expressed in mouse liver. Interestingly, the mRNA expression levels of MGAT5B, MGAT6 and EDEM3 were reduced in comparison to GMIIx and the other N-glycan processing ensymes. GMIIx trancript expression was also examined in various mammalian cell lines by qRT-PCR (Fig. 2.9). GMIIx was transcribed at similar levels in MCF-7 breast cancer cells, HEK293 cells, and HEK293 cells transfected with GMII, GMIIx_v1a or GMIIx_v2b. It was also found that GMIIx transcripts are expressed at reduced levels in HeLa cells, however, the significance of this data was not examined in detail.

Construction of an Expression Vector Encoding Human Golgi α-Mannosidase IIx

Human Golgi α-mannosidase IIx has been partially characterized in previous studies leading to ambiguities about its function *in vivo*. In addition, several sequence variants were identified among cloned cDNAs, but detailed characterization of the relative catalytic activities of the recombinant products had not been performed. In order to resolve this issue, expression plasmids encoding the various splice isoforms of GMIIx catalytic domains were constructed. Expressed sequence tags (EST) encoding regions of the GMIIx splice variants were compared at the protein sequence level and the sequence alignment showed the presence of two putative intervening sequences, IS1 and IS2. Additionally, three locations where the ESTs contain putative base insertions or deletions were identified, insertion/deletion sequence 1 (I/D 1), insertion/deletion sequence 2 (I/D 2), and insertion/deletion sequence 3 (I/D 3). Several of the EST

sequences were assembled as expression constructs for recombinant production in HEK293 cells. In addition, a cDNA sequence was assembled from the respective ESTs that contained all of the protein sequence elements with greatest similarity to GMII and mouse GMIIx. To assemble this latter construct, the 3' end of the GMIIx open reading frame spanning the SacI-NotI sites (Fig 2.10) was replaced with the corresponding sequence from a human brain cDNA clone, GMIIx_v1a/2b. This removed an intervening sequence (IS1) that led to the premature termination of translation and a truncated polypeptide. However, the cDNA contained a previously uncharacterized intervening sequence (IS2). IS2 was removed by swapping the 3' end of GMIIx_v1a/v2b into the corresponding region of GMIIx_v1b/2a producing GMIIx_v1a/2a and the end product was completely sequenced (Fig. 2.10).

Recombinant Protein Expression in HEK293 Cells

The three splice variants of GMIIx, GMIIx_v1b/2a, GMIIx_v1a/2b and GMIIx_v1a/2a with the reassembled catalytic domain sequence with greatest similarity to mouse GMIIx were stably transfected into HEK293 cells and the conditioned media was assayed for mannosidase activity (Fig. 2.11) The melanoma cDNA variant of GMIIx, GMIIx_v1b/2a, expressed little functional protein, consistent with our earlier attempts to express GMIIx. Interestingly, GMIIx_v1a/2b expressed a protein with moderate levels of mannosidase activity as compared to HEK293 cells expressing hGMII. In addition, the recombinant, catalytic domain of GMIIx had higher mannosidase activity toward the 4-MU α-Man substrate when compared to recombinant human GMII.

Purification of Recombinant Human Golgi α-Mannosidase IIx

Based on the initial assay data, the soluble, recombinant form of human Golgi α-mannosidase IIx was purified from HEK293 cells stably transfected with GMIIx_v1a/2a. Briefly, conditioned media from the transfected HEK293 cells was harvested and processed using a purification strategy employing phenyl-Sepharose (Fig. 2.12), blue-Sepharose (Fig. 2.13), and hydroxyapatite chromatographies (Fig. 2.14). Table 2.1 shows the results of a typical purification protocol. Enzyme activity was tracked using a 4-MU α-Man assay during each of the purification steps. It should be noted that strategies employing purification with the His tag or the HA tag were unable to affinity purify the recombinant protein. This suggests that the N-terminal end of the secreted protein may have been lost through proteolysis during expression or purification. It remains an open question whether proteolysis is occurring *in vitro*. Another possible reason for the inability of recombinant GMIIx to be purified by affinity chromatography is the presence of contaminating proteins present in the conditioned media that inhibit binding of GMIIx protein to the affinity column. To confirm the identity of the expressed protein, the purified enzyme was resolved by SDS-PAGE, stained with Coomassie G-250, excised, digested with trypsin and sequenced by liquid chromatography/tandem mass spectrometry. The expressed protein had a specific activity of 1.2 units/mg (Table 2.1) and a size of ~ 125 kDa as determined by SDS-PAGE, consistent with other class II α-mannosidases and sequence data confirmed the identity of the recombinant product (Fig. 2.15).

Biochemical Characterization of Recombinant Human Golgi α-Mannosidase IIx

In order to determine whether recombinant GMIIx is sensitive to the presence of cations, the purified rGMIIx enzyme was assayed in the presence of $CoCl_2$, $CaCl_2$, $ZnCl_2$, $NiCl_2$ and $FeCl_2$ (Fig. 2.16). Cobalt ions stimulate rGMIIx activity 2.5 fold, whereas the other ions have no effect. Assays performed at a range of pH values indicated that rGMIIx has a pH optimum at 5.6, consistent with the localization of GMIIx to the cis- to medial- Golgi apparatus (Fig. 2.17). rGMIIx was assayed with increasing concentrations of 4-MU α-Man and the data was plotted in a double reciprocal Lineweaver-Burk plot (Fig. 2.18). Linear regression of the data indicated that rGMIIx has a K_m of 10.7 mM and a V_{max} of 5.4 nmol/min using 4-MU α-Man as a substrate (Table 2.2). The K_m value for rGMIIx is consistent with previous kinetic studies of α-mannosidases with a synthetic substrate (Table 2.3). Slightly higher K_m values employing synthetic substrates versus native oligosaccharide substrates suggests that α-mannosidases are less specific for synthetic substrates. rGMIIx is sensitive to inhibition by swainsonine (SW) and 1,4-dideoxy-1,4-imino-D-mannitol (DIM), but not to kifunensine (KIF) and deoxymannojirimycin (DMJ) (Table 2.4). As found with other GH38 α-mannosidases, rGMIIx is sensitive to DIM inhibition in the micromolar range, whereas the enzyme is sensitive to SW inhibition in the submicromolar range (Table 2.4). Collectively, these data confirm that rGMIIx has many of the characteristics of class II α-mannosidases.

Substrate Specificity of Golgi α-Mannosidase IIx

Purified recombinant Golgi α-mannosidase IIx was assayed with pyridylamine (PA)-tagged oligosaccharide substrates to determine its relative activity toward natural substrate glycans. In a time course study, rGMIIx was unable to cleave the high mannose oligosaccharides $Man_{9-5}GlcNAc_2$-PA, but following prolonged 24 hour digestion, the enzyme was able to hydrolyze $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_4GlcNAc_2$-PA and $GlcNAcMan_3GlcNAc_2$-PA (Fig. 2.19). To confirm that rGMIIx has a similar substrate specificity as GMII, the enzymes were immunoprecipitated from either the conditioned media or cell lysates of HEK293 cells transfected with either GMII or rGMIIx. The enzymes were assayed with the oligosaccharides $GlcNAcMan_5GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA. Both enzymes were able to cleave $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_3GlcNAc_2$-PA, however, neither enzyme hydrolyzed the high mannose oligosaccharides $Man_{9-5}GlcNAc_2$-PA.

To confirm whether the difference in substrate specificity for our recombinant GMIIx catalytic domain construct was different from the activity reported earlier (Oh-Eda, et al., 2001) for a different recombinant splice variant, we prepared membrane fractions from wild-type (wt) mouse liver, GMII null mouse liver, GMIIx null mouse liver and GMII/GMIIx heterozygous mice livers. Crude homogenate, cytosol, microsomal/Golgi membranes and lysosomal/mitochondrial membranes from wt mouse liver were immunoprecipitated with antibodies against GMII, GMIIx, or the cytosolic α-mannosidase (CytMan). Assaying the immunoprecipitates with natural $GlcNAcMan_5GlcNAc_{1-2}$-PA oligosaccharides showed that GMII is mainly localized to the

microsomal/Golgi membranes and there is no difference between the cleavage pattern found in HEK 293 cells and wt mouse liver nor between substrates with one or two GlcNAc residues on the reducing end (Table 2.5). No GMII immunoprecipitated activity towards the oligosaccharide substrates was detected in mice where GMII protein expression was abrogated by genetic disruption. In contrast to the results obtained with GMII, no activity was detected in any mouse tissue fractions with the GMIIx antibody (Table 2.6). There are four possible explanations for these results. First, the antibody employed has been tested and confirmed for immunoprecipitation of human rGMIIx, but the antibody might not immunoprecipitate the mouse protein. Inability to immunoprecipitate the mouse enzyme would lead to a false negative in assays following the immunoprecipitation. Alternatively, the antibody could mask the active site of GMIIx preventing any hydrolysis of the oligosaccharide substrate. Another possibility is that the amount of GMIIx protein found in tissue fractions is too low to be detected by immunoprecipitation. A fourth possibility is that GMIIx is a labile protein *in vivo*. Assaying microsomal/Golgi membranes from GMII null mouse livers with $GlcNAcMan_5GlcNAc_2$-PA resulted in no cleavage of the native oligosaccharide substrate, providing evidence for this possibility.

To verify that the results were specifically due to the presence of GMII or the absence of GMIIx, mouse liver fractions were immunoprecipitated with the cytosolic α-mannosidase antibody and assayed with PA-tagged oligosaccharides. A cobalt-dependent activity immunoprecipitated by the cytosolic α-mannosidase antibody hydrolyzed $Man_5GlcNAc$-PA to $Man_4GlcNAc$-PA and was localized to the cytosol and microsomal/Golgi fractions of wt mouse livers (Table 2.7). Interestingly, the PA-tagged

oligosaccharide $Man_{6A}GlcNAc_2$-PA was hydrolyzed to $Man_5GlcNAc_2$-PA in mouse liver microsomal/Golgi membranes in the presence of 5 mM cobalt without immunoprecipitation with any of the α-mannosidase antibodies. These data indicate that the cytosolic α-mannosidase could account for the low levels of $Man_5GlcNAc$ cleaving activity that was detected in GMII null mice. Whether this enzyme is truly an intrinsic lumenal activity in the secretory pathway or merely a low level contaminant from the cytosol adhering to the microsomal membrane fraction remains to be resolved. The fact that GMII/GMIIx double null mice have a severe lethal pathology and lose the capacity to produce complex type glycans indicates that the cytosolic α-mannosidase does not contribute to a functional glycan processing pathway *in vivo*.

DISCUSSION

Class II (GH 38) mannosidases are identified by their structural and functional properties including substrate specificity, divalent cation requirements, pH optimum, sensitivity to inhibition, size and catalytic mechanism. Golgi α-mannosidase IIx has been identified as a member of this family without clear data regarding its biological role. Here, we report that human GMIIx is a member of CAZy family GH 38 based on its specificity for the hybrid type oligosaccharide $GlcNAcMan_5GlcNAc_2$, activation by Co^{2+}, pH optimum of 5.5-6.0, sensitivity to the inhibitors swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol, and molecular weight of approximately 125 kDa. It is assumed that GMIIx cleaves the terminal α1→3 and α1→6 mannose residues on $GlcNAcMan_5GlcNAc_2$ to generate $GlcNAcMan_3GlcNAc_2$.

Much attention has been drawn to the possibility that GMIIx catalyzes an alternate pathway for complex type N-glycan formation bypassing the pathway utilized by GMII. This pathway was originally proposed based on evidence that CHO cells transfected with full length GMIIx show a reduction in $Man_6GlcNAc_2$ and an increase in $Man_4GlcNAc_2$, implying GMIIx is catalyzing the conversion. Studies based on the structure of GMII have highlighted residues important for substrate and inhibitor binding. In these studies, based on the crystal structure of Dm GMII, a model is proposed for substrate binding and catalysis. Three groups of amino acid residues are important for the catalytic mechanism, the inhibitor binding residues D472, R228 and R876, the conserved catalytic aspartates, D204, D341 and Y267, and the GlcNAc binding residues, W299, P298 and H273 (Fig. 2.20) (Shah, *et al.*, 2008). It is concluded that the presence on the GlcNAc binding residues allows selective catalysis of the hybrid type oligosaccharide substrate $GlcNAcMan_5GlcNAc_2$ whereas the high mannose oligosaccharide $Man_5GlcNAc_2$ binds to the active site with no subsequent catalysis due to inappropriate spatial localization of the catalytic amino acid residues with the oligosaccharide (Shah, *et al.*, 2008). Upon examination of the full length sequence of GMIIx, it becomes evident that the protein contains all of the amino acid residues necessary for catalysis of the hybrid type oligosaccharide $GlcNAcMan_5GlcNAc_2$ (Fig. 2.2). A study examining the catalytic specificity of Dm GMII showed that the enzymes hydrolyzes the oligosaccharide $GlcNAcMan_5GlcNAc_2$ with an 80 fold preference over $Man_5GlcNAc_2$ (Zhong, *et al.*, 2008).

In light of the structural information gained by analyzing the catalytic mechanism of Dm GMII, functional studies of GMIIx in animal models and biochemical

characterization of the catalytic domain of the murine variant of GMIIx, the functional role of GMIIx in degrading high mannose oligosaccharides is now clear. Our results show that GMIIx is unable to cleave PA-tagged high mannose oligosaccharides and hydrolyzes the hybrid type oligosaccharide $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_3GlcNAc_2$-PA. This is consistent with the conclusions drawn from analysis of the GMII/GMIIx double null mouse where it was shown that GMII and GMIIx catalyze functionally redundant pathways in a tissue-specific manner.

We attempted to verify the model for processing of hybrid type oligosaccharides by immunoprecipitation of GMIIx protein in mouse tissues. However, we were unable to detect any expressed protein in mouse liver or kidney either in crude tissue extracts or following immunoprecipitation. While it is possible that the antibody to human GMIIx did not cross react with the murine ortholog, it is also likely that the amount of GMIIx protein being expressed in these tissues is too low to analyze biochemically with current technologies. RT-PCR of transcript expression in mammalian cell lines and human tissues showed that GMIIx mRNA is being expressed in the mammalian cell lines and in human muscle, spleen and testis. It would be interesting to examine the relative protein expression levels of GMIIx among diverse species, including mice and humans.

In contrast to GMIIx, we detected an activity in mouse liver microsomal/Golgi membranes and cytosol that hydrolyzes $Man_5GlcNAc$-PA to $Man_4GlcNAc$-PA in the presence of 5 mM Co^{2+}. This confirms the presence of a detectable activity *in vitro* that previously led to the hypothesis of an alternative bypass pathway. However, we found no evidence that GMIIx is responsible for catalyzing this activity and suggestive evidence that the cytosolic α-mannosidase can account for the *in vitro* hydrolysis of this

substrate in membrane fractions. These data indicate that the activity previously attributed to a "Golgi α-mannosidase III" is likely an *in vitro* artifact of membrane fractionation and that no alternative bypass activity exists in animal cells for N-glycan processing beyond the functionally redundant activities of GMII and GMIIx.

MATERIALS AND METHODS

Buffers and Solutions

Buffer A: 50 mM phosphate buffer, 50% 400 mM ammonium sulfate, pH 7.5. Buffer B: 50 mM phosphate buffer, 50% ethylene glycol, pH 7.5. Buffer C: 20 mM phosphate buffer, pH 7.5. Buffer D: 1 M NaCl. Buffer E: 200 mM phosphate buffer, pH 7.5.

Mannosidase Assays

Samples were analyzed for mannosidase activity using synthetic or natural oligosaccharide substrates. Mannosidase activity was assayed using the synthetic substrate 4-methylumbelliferyl-α-D-mannopyranoside (4-MU α-Man) (Sigma). Briefly, 5 μL of sample was assayed with 5 mM 4-MU α-Man in 100 mM sodium acetate, pH 5.6 in a total reaction volume of 50 μL at 37°C for 30-60 min. The reaction was stopped by the addition of 200 μL 0.2 M sodium bicarbonate to a final concentration of 160 mM. Product formation was determined by quantitating the fluorescence at 360 nm

excitation and 455 nm emission using a Spectramax Gemini XS fluorescence reader. All fluorescence values were compared with a 4-MU α-Man standard curve. Mannosidase activity using natural oligosaccharide substrates was assayed as follows. High mannose- and hybrid-type pyridylamine (PA)-tagged oligosaccharides including $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, $Man_5GlcNAc_2$, and $GlcNAcMan_5GlcNAc_2$ were prepared as described previously (Karaveg, *et al.*, 2005). $Man_5GlcNAc$ was prepared by endoglycosidase H digestion of $Man_5GlcNAc_2$. $GlcNAcMan_5GlcNAc$ was prepared by incubating $Man_5GlcNAc$ with GNTI (expression constructs kindly provided by Dr. Harry Schachter). The product, $GlcNAcMan_5GlcNAc$, was isolated by reverse-phase HPLC as described previously (Liao, *et al.*, 1996, Lal, *et al.*, 1998). A 5 μL sample was incubated with 10-200 nmol of oligosaccharide in 100 mM sodium acetate, pH 5.6 in a total reaction volume of 50 μL at 37°C for 30 min. Reactions were stopped by heat inactivation at 95°C for 5 min and extracted with 1:1 phenol:chloroform twice. Product formation was detected by HPLC on an amine APS-2 hypersil column (Waters). Assays with GMIIx were performed in the presence of 5 mM Co^{2+}. Assays with the cytosolic α-mannosidase were performed in 0.1 M MES buffer at pH 6.5 and in the presence of 5 mM 5 mM Co^{2+}. Assays with the broad-specificity lysosomal α-mannosidase were performed in 0.1 M sodium acetate buffer at pH 4.0.

Cell Culture

Human embryonic kidney (HEK293) cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) (Sigma) and 10% calf serum (Sigma) in 5% CO_2 at

37°C on 75 cm² tissue culture flasks (Corning). For the generation of stable transfectants, the cells were grown to 50-80% confluency in DMEM and 10% calf serum at 37°C. The cells were transfected according to the calcium phosphate transfection protocol. Briefly, 30 μg of plasmid DNA were added to 0.5 mL of 1X HEPES buffered saline followed by mixing. 30 μL of 2.5 M $CaCl_2$ was added to the transfection mixture, mixed and allowed to sit for 20 minutes. The transfection mixture was added in a dropwise manner to HEK293 cells at 50% confluency and the reaction was placed in 37°C, 5% CO_2 overnight. The following day, the media was replaced with fresh DMEM. Transfected cells were placed in puromycin selection. The cells were grown at 37°C overnight prior to selection with 1.0 μg/ml puromycin. After reaching confluency, the cells were split and the antibiotic concentration was increased to 2.0 μg/ml. For enzyme production, the transfected cells were grown in T-175 flasks in DMEM and 10% calf serum at 37°C until reaching confluency. HeLa cells and MCF-7 breast cancer cells were a kind gift from Dr. Michael J. Pierce, University of Georgia and maintained as described above.

Sequence Alignment of Class II Golgi α-mannosidases

The translation products of GMII (GenBank accession number NM_002372.2) and the GMIIx transcripts, GMIIx_v1a (GenBank accession number NM_006122.2), GMIIx_v1b (GenBank accession number L28821.1), GMIIx_v2b (GenBank accession number BI758056.1) and GMIIx_v3b (GenBank accession number D55649.1) were

aligned using the the program ClustalW. The alignment output was transformed into an image using the program SeaView 4.

Construction of GMIIx Splice Variant Expression Vectors

Recombinant human Golgi α-mannosidase IIx was assembled in the pEAK10 expression vector (Edge Biosystems). The GMIIx cDNA from a human melanoma library was subcloned into pBSSK+ downstream of a T. cruzi signal sequence, a 8x His tag and a Haemophilus influenza (HA) epitope tag and was name GMIIx_v1b/2a. The 5' end of the open reading frame from GMIIx cDNA spanning the BamHI site to the SacI site was isolated with restriction endonucleases. The 3' end was generated by PCR from a human brain cDNA IMAGE clone 5200846 (Invitrogen). The primers used were MIIxFront30: CAGCCCCTGGCCGTGCAGATCAGCGCACAC and MIIxStopNotI: ATAGTTTAGCGGCCGCCTAACCCAAGCGGAGGCGAAAGGTAGCAAT. The PCR product was digested with SacI and NotI. The two fragments were subcloned together into pEAK10 using the BamHI and NotI restriction sites. This construct contains intervening sequence 2 (IS2) and was named GMIIx_v1a/2b. The 3' end of the open reading frame from the BlnI site to the Not I site was swapped with the identical region from the GMIIx melanoma cDNA. The final product was named GMIIx_v1a/2a.

Phenyl Sepharose Chromatography

Conditioned media (2L) from HEK293 cells stably transfected with recombinant human GMIIx were harvested and clarified by centrifugation at 3000 rpm for 10 min. Ammonium sulfate was added to a final concentration of 400 mM. The media was loaded onto a phenyl Sepharose column (Amersham Biosciences) pre-equilibrated in buffer A. rGMIIx was eluted from the column using a step gradient of 140 ml buffer A, 375 mL 0-100% buffer B, and 375 mL buffer B at a flow rate of 6.0 ml/min. 9 mL fractions were collected from the elution range 170-890 mL and assayed for mannosidase activity as described above. Protein concentration in the fractions was determined using the BCA assay kit (Pierce). Fractions containing active rGMIIx were pooled.

Blue Sepharose Chromatography

Pooled fractions from the phenyl sepharose chromatography step were dialyzed in 4L of buffer C overnight at 0^{o}C. The dialyzed sample was loaded onto a blue sepharose 6L-CB (Sigma) column pre-equilibrated in buffer C. rGMIIx was eluted with a step gradient of 10 mL buffer C, 160 mL 0-100% buffer D, and 10 mL buffer D. Fractions of 1 mL were collected and assayed for mannosidase activity as described above. Protein concentration was determined using the BCA assay kit (Pierce). Fractions containing active rGMIIx were pooled.

Ion exchange chromatography

The pooled fractions from the blue sepharose chromatography step was diluted to 500 mL with buffer C and loaded onto a hydroxyapatite CHT2 column (BioRad) pre-equilibrated in buffer C. rGMIIx was eluted using a step gradient of 10 mL buffer C, 200 mL 0-100% buffer E, and 10 mL buffer E. Fractions of 1 mL were collected and assayed for mannosidase activity as described above. Protein concentration was determined using the BCA assay kit (Pierce). Fractions containing active rGMIIx were pooled and stored in 50% glycerol.

Identification of Purified Recombinant Golgi α-Mannosidase IIx by SDS-PAGE and Mass Spectrometry

The identity of purified rGMIIx was verified by SDS-PAGE and mass spectrometry as described previously (Park, *et al.*, 2006).

Divalent cation, pH and inhibitor studies

The effect of divalent cations on rGMIIx activity was determined by assaying the purified enzyme in the presence of 5 mM $CoCl_2$, $ZnCl_2$, $CaCl_2$, $NiCl_2$, $FeCl_2$. The pH dependence curve was determined using buffers comprised of 100 mM sodium acetate over the pH range of 4-5.6, 100 mM bis-tris buffer over the pH range of 6-6.5, and 100 mM Tris was used for pH 7-8. For the inhibitor studies, swainsonine (SW), 1,4-

dideoxy-1,4-imino-D-mannitol (DIM), kifunensine (KIF), and 1-deoxymannojirimycin were prepared as stock solutions in water. Lineweaver-Burk plots and Dixon plots were used to transform the kinetic data into the kinetic constants, K_m and K_i as described previously (Segel, 1993).

Substrate Specificity of Golgi α-Mannosidase IIx

The substrate specificity of GMIIx was determined by assaying mammalian cell lines and membrane fractions from mouse livers with natural oligosaccharide substrates. For mammalian cell lines, cell lysates were prepared by harvesting confluent cells, washing the cells three times in phosphate-buffered saline (PBS), and lysing the cells in 1% Triton X-100/PBS. Conditioned media or cell lysates of HEK293 cells transfected with GMII or GMIIx were immunoprecipitated with protein A-agarose beads (Repligen) bound to anti-rat Golgi α-mannosidase II (α-RGMII) or anti-human Golgi α-mannosidase IIx (α-GMIIx) antibodies and incubated overnight at 0°C. The anti-human Golgi α-mannosidase IIx antibody was generated by immunizing a rabbit with 0.5 mg of purified recombinant GMIIx (Animal Care Facility, Life Sciences Building, University of Georgia). The HGMIIx antibody specifically bound recombinant HGMIIx protein as determined by Western blotting and 4-MU α-Man assays of immunoprecipitated recombinant HGMIIx. The immunocomplexes were washed three times in PBS and assayed with PA-tagged oligosaccharides. Product formation was determined by HPLC. Membrane fractions from wild type C57/bl mouse livers were prepared and all of the steps were performed on ice. Mouse livers were homogenized in 5 mL of 0.5 M sucrose

with a Polytron homogenizer at 5-6 for 15-20s. The homogenate was centrifuged at 3000 rpm for 12 min. The supernatant was saved and the nuclear pellet was resuspended in 5 mL of 0.5 M sucrose. The pellet was homogenized with a Polytron homogenizer at 5-6 for 10-15 s and centrifuged at 3000 rpm for 12 min. The supernatants were pooled and layered onto a 15 mL cushion of 1.3 M sucrose. The sucrose gradient was centrifuged at 45000 rpm for 90 min. The supernatant (cytosol) was saved and the interface (microsomal/Golgi membranes) was washed two times in 0.4 M PBS. The resultant pellet was solubilized two times with 1.0% Triton X-100/PBS and once with 1.0% Triton X-100/0.5 M NaCl/PBS. The solubilized fractions were pooled and assayed with PA-tagged oligosaccharides. C57/bl mouse liver was a kind gift from Dr. J. Michael Pierce, University of Georgia, GA. GMII null, GMIIx and GMII/ GMIIx heterozygous mouse livers were a kind gift from Dr. Tomoya Akama, the Burnham Institute, CA.

Transcript Analysis of GMIIx in Human Tissues by RT-PCR

The expression of GMII or GMIIx transcripts in mammalian cell lines were performed as described previously (Nairn, *et al.*, 2008). The presence of intervening sequences IS1 or IS2 in GMIIx transcripts was analyzed by RT-PCR. The primers used for IS1 were: 5' MIIx 2375 CAGGTCTGGTTCTCAGGCCTTACTGGGCTC, 3' MIIx 2445 TCCCACCTACTCCTCGTGCTCGTCGTCCAC and the primers used for IS2 were 5' MIIx 2745 TGGCCCTGCACATCCATACAGACATCGACA and 3' MIIx 2825 TGCCATAGACTTCTTCGAGGGGGAGGTCCG. First strand cDNA was prepared from 5

μg total RNA or 500 ng mRNA from human muscle, spleen or testis and 10 μM primer using the Superscript III reverse transcriptase (Invitrogen). The cDNA was amplified by PCR using either program 1: amplification for 30 cycles, 30 s @ 94°C, 15 s @ 65°C, and 30 s @ 72°C, or program 2: amplification for 30 cycles, 30 s @ 94°C and 30 s @ 72°C.

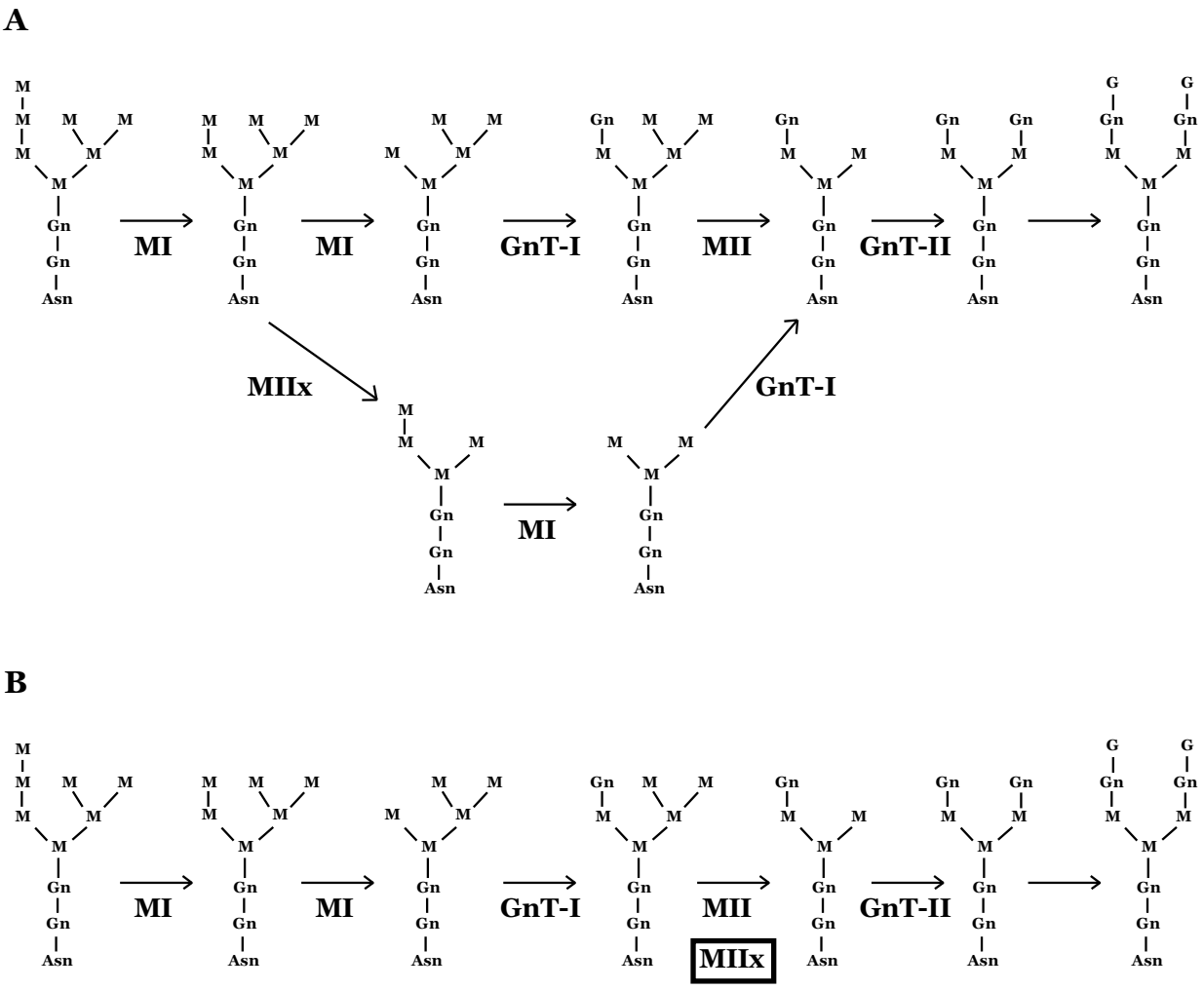
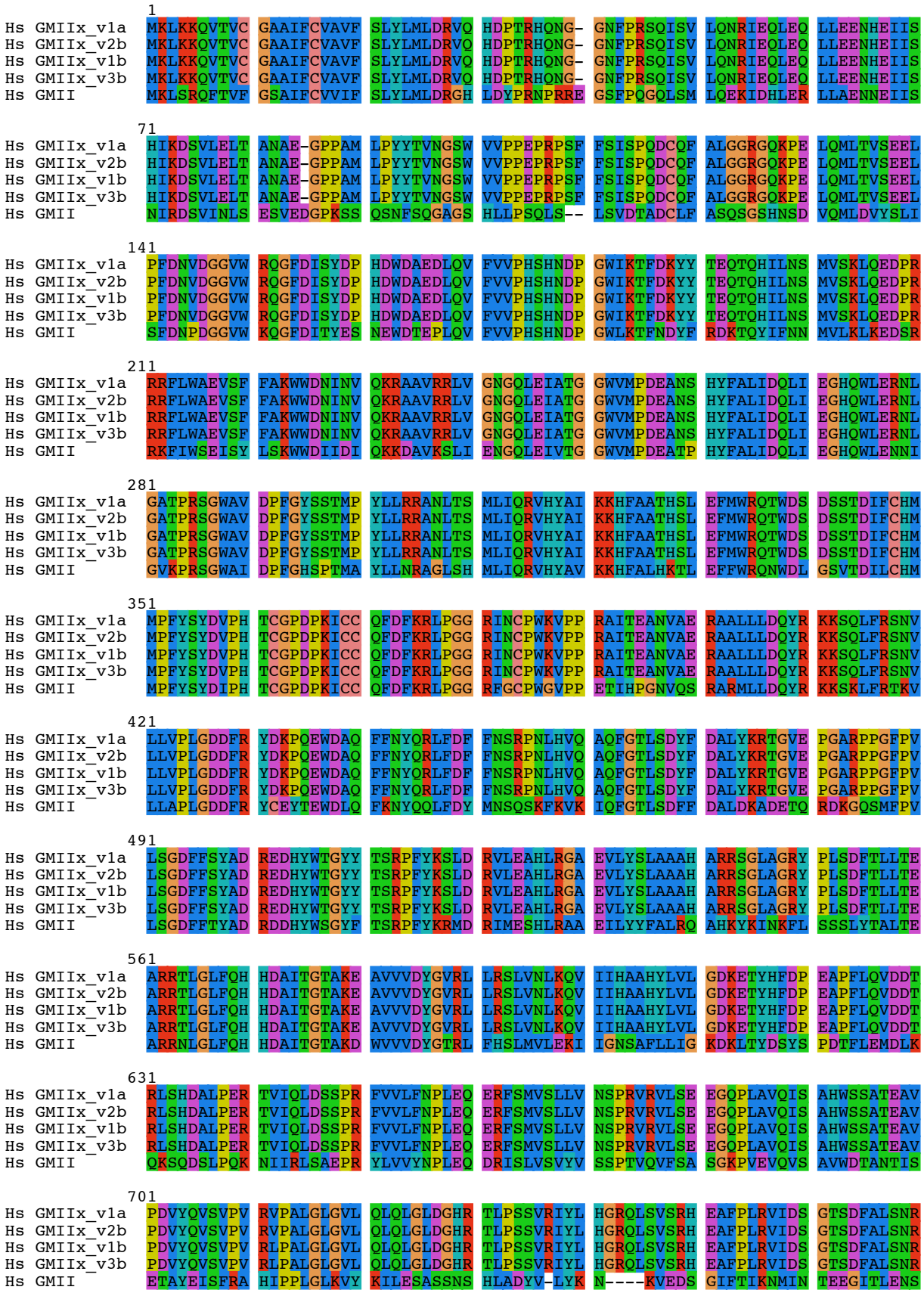


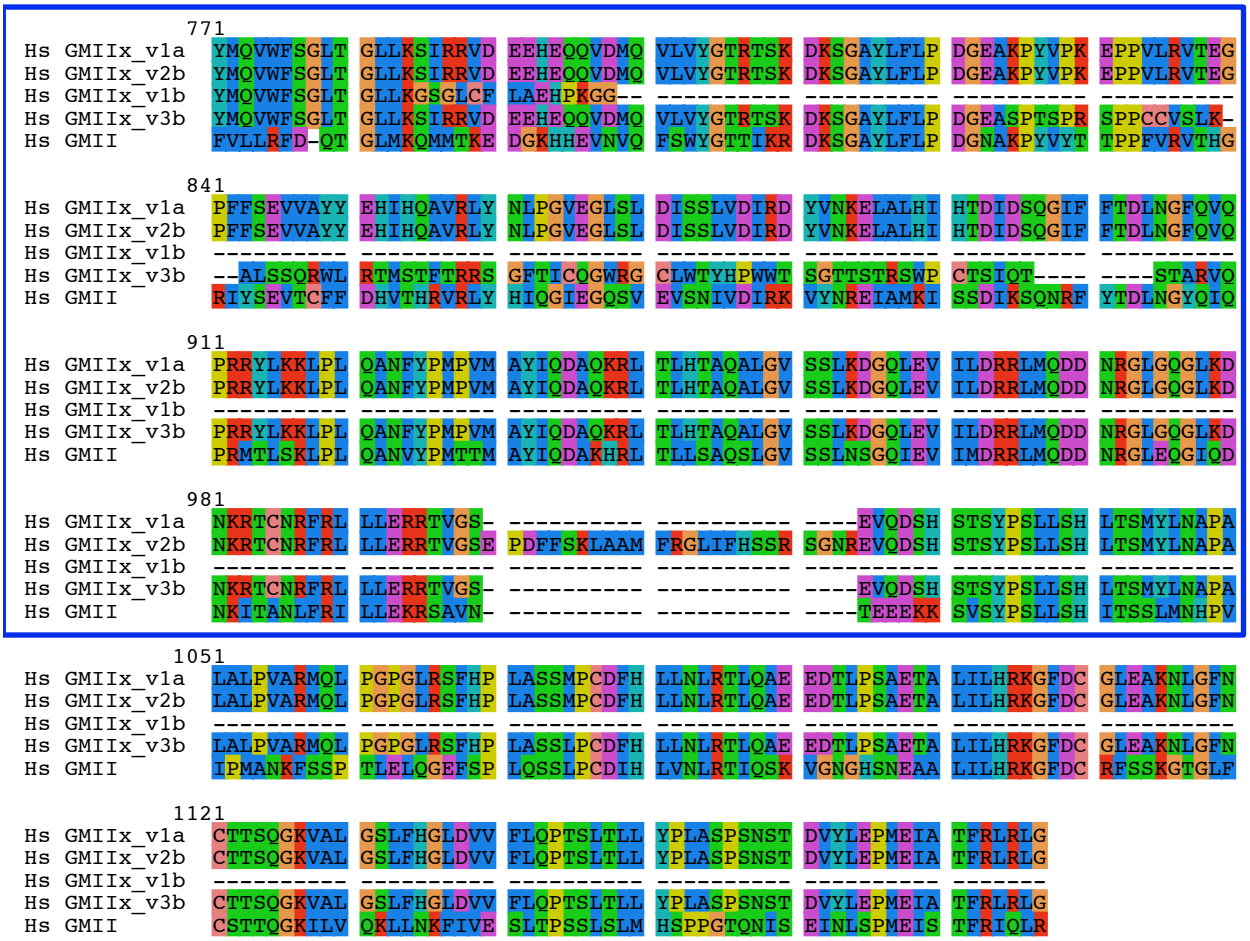
Fig. 2.1. Models of N-glycoprotein processsing in the Golgi apparatus. (A) A model where GMIIx catalyzes an alternative pathway to complex N-glycoprotein biosynthesis bypassing GMII. (B) An alternative model where GMII and GMIIx catalyze functionally redundant pathways. Abbreviations for enzyme activities are: MI, Golgi α-mannosidase I; GnT-I, N-acetylglucosaminyltransferase I; MII, Golgi α-mannosidase II; MIIx, Golgi α-mannosidase IIx; GnT-II, acetylglucosaminyltransferase II.

Oh-Eda, et al. (2001). *Eur J Biochem* **268**:1280-1288.,

Akama, et al. (2006). *Proc Natl Acad Sci U S A* **103**(24):8983-8988.

Fig. 2.2. The translation products of various GMIIx mRNA splice variants were aligned at the protein sequence level. The GMIIx mRNA sequences used for the alignment are: GMIIx_v1a, a variant expressed in human lymphocytes (NM_006122.2); GMIIx_v1b, a variant expressed in human melanoma cells (L28821.1); GMIIx_v2b, a variant expressed in human brain tissues (BI758056.1); GMIIx_v3b, a variant expressed in human melanoma cells (D55649.1); and Hs GMII, human Golgi α-mannosidase II (NM_002372.2). The splice variants show extensive regions of similarity near the N-terminus which contains residues important for catalysis whereas the C-terminus contains a region of protein sequence divergence as indicated by the blue box. The protein sequences were aligned with ClustalW and displayed with SeaView 4.


1
Hs GMIIx_v1a MKLKKQVTVC GAAIFCVAVF SLYLMLDRVQ HDPTRHQNG- GNFPRSQISV LQNRIEQLEQ LLEENHEIIS
Hs GMIIx_v2b MKLKKQVTVC GAAIFCVAVF SLYLMLDRVQ HDPTRHQNG- GNFPRSQISV LQNRIEQLEQ LLEENHEIIS
Hs GMIIx_v1b MKLKKQVTVC GAAIFCVAVF SLYLMLDRVQ HDPTRHQNG- GNFPRSQISV LQNRIEQLEQ LLEENHEIIS
Hs GMIIx_v3b MKLKKQVTVC GAAIFCVAVF SLYLMLDRVQ HDPTRHQNG- GNFPRSQISV LQNRIEQLEQ LLEENHEIIS
Hs GMII MKLSRQFTVF GSAIFCVVIF SLYLMLDRGH LDYPRNPRRE GSFPQGQLSM LQEKIDHLER LLAENNEIIS
71
Hs GMIIx_v1a HIKDSVLELT ANAE-GPPAM LPYYTVNGSW VVPPEPRPSF FSISPQDCQF ALGGRGQKPE LQMLTVSEEL
Hs GMIIx_v2b HIKDSVLELT ANAE-GPPAM LPYYTVNGSW VVPPEPRPSF FSISPQDCQF ALGGRGQKPE LQMLTVSEEL
Hs GMIIx_v1b HIKDSVLELT ANAE-GPPAM LPYYTVNGSW VVPPEPRPSF FSISPQDCQF ALGGRGQKPE LQMLTVSEEL
Hs GMIIx_v3b HIKDSVLELT ANAE-GPPAM LPYYTVNGSW VVPPEPRPSF FSISPQDCQF ALGGRGQKPE LQMLTVSEEL
Hs GMII NIRDSVINLS ESVEDGPKSS QSNFSQGAGS HLLPSQLS-- LSVDTADCLF ASQSGSHNSD VQMLDVYSLI
141
Hs GMIIx_v1a PFDNVDGGVW RQGFDISYDP HDWDAEDLQV FVVPHSHNDP GWIKTFDKYY TEQTQHILNS MVSKLQEDPR
Hs GMIIx_v2b PFDNVDGGVW RQGFDISYDP HDWDAEDLQV FVVPHSHNDP GWIKTFDKYY TEQTQHILNS MVSKLQEDPR
Hs GMIIx_v1b PFDNVDGGVW RQGFDISYDP HDWDAEDLQV FVVPHSHNDP GWIKTFDKYY TEQTQHILNS MVSKLQEDPR
Hs GMIIx_v3b PFDNVDGGVW RQGFDISYDP HDWDAEDLQV FVVPHSHNDP GWIKTFDKYY TEQTQHILNS MVSKLQEDPR
Hs GMII SFDNPDGGVW KQGFDITYES NEWDTEPLQV FVVPHSHNDP GWLKTFNDYF RDKTQYIFNN MVLKLKEDSR
211
Hs GMIIx_v1a RRFLWAEVSF FAKWWDNINV QKRAAVRRLV GNGQLEIATG GWVMPDEANS HYFALIDQLI EGHQWLERNL
Hs GMIIx_v2b RRFLWAEVSF FAKWWDNINV QKRAAVRRLV GNGQLEIATG GWVMPDEANS HYFALIDQLI EGHQWLERNL
Hs GMIIx_v1b RRFLWAEVSF FAKWWDNINV QKRAAVRRLV GNGQLEIATG GWVMPDEANS HYFALIDQLI EGHQWLERNL
Hs GMIIx_v3b RRFLWAEVSF FAKWWDNINV QKRAAVRRLV GNGQLEIATG GWVMPDEANS HYFALIDQLI EGHQWLERNL
Hs GMII RKFIWSEISY LSKWWDIIDI QKKDAVKSLI ENGQLEIVTG GWVMPDEATP HYFALIDQLI EGHQWLENNI
281
Hs GMIIx_v1a GATPRSGWAV DPFGYSSTMP YLLRRANLTS MLIQRVHYAI KKHFAATHSL EFMWRQTWDS DSSTDIFCHM
Hs GMIIx_v2b GATPRSGWAV DPFGYSSTMP YLLRRANLTS MLIQRVHYAI KKHFAATHSL EFMWRQTWDS DSSTDIFCHM
Hs GMIIx_v1b GATPRSGWAV DPFGYSSTMP YLLRRANLTS MLIQRVHYAI KKHFAATHSL EFMWRQTWDS DSSTDIFCHM
Hs GMIIx_v3b GATPRSGWAV DPFGYSSTMP YLLRRANLTS MLIQRVHYAI KKHFAATHSL EFMWRQTWDS DSSTDIFCHM
Hs GMII GVKPRSGWAI DPFGHSPTMA YLLNRAGLSH MLIQRVHYAV KKHFALHKTL EFFWRQNWDL GSVTDILCHM
351
Hs GMIIx_v1a MPFYSYDVPH TCGPDPKICC QFDFKRLPGG RINCPWKVPP RAITEANVAE RAALLLDQYR KKSQLFRSNV
Hs GMIIx_v2b MPFYSYDVPH TCGPDPKICC QFDFKRLPGG RINCPWKVPP RAITEANVAE RAALLLDQYR KKSQLFRSNV
Hs GMIIx_v1b MPFYSYDVPH TCGPDPKICC QFDFKRLPGG RINCPWKVPP RAITEANVAE RAALLLDQYR KKSQLFRSNV
Hs GMIIx_v3b MPFYSYDVPH TCGPDPKICC QFDFKRLPGG RINCPWKVPP RAITEANVAE RAALLLDQYR KKSQLFRSNV
Hs GMII MPFYSYDIPH TCGPDPKICC QFDFKRLPGG RFGCPWGVPP ETIHPGNVQS RARMLLDQYR KKSKLFRTKV
421
Hs GMIIx_v1a LLVPLGDDFR YDKPQEWDAQ FFNYQRLFDF FNSRPNLHVQ AQFGTLSDYF DALYKRTGVE PGARPPGFPV
Hs GMIIx_v2b LLVPLGDDFR YDKPQEWDAQ FFNYQRLFDF FNSRPNLHVQ AQFGTLSDYF DALYKRTGVE PGARPPGFPV
Hs GMIIx_v1b LLVPLGDDFR YDKPQEWDAQ FFNYQRLFDF FNSRPNLHVQ AQFGTLSDYF DALYKRTGVE PGARPPGFPV
Hs GMIIx_v3b LLVPLGDDFR YDKPQEWDAQ FFNYQRLFDF FNSRPNLHVQ AQFGTLSDYF DALYKRTGVE PGARPPGFPV
Hs GMII LLAPLGDDFR YCEYTEWDLQ FKNYQQLFDY MNSQSKFKVK IQFGTLSDFF DALDKADETQ RDKGQSMFPV
491
Hs GMIIx_v1a LSGDFFSYAD REDHYWTGYY TSRPFYKSLD RVLEAHLRGA EVLYSLAAAH ARRSGLAGRY PLSDFTLLTE
Hs GMIIx_v2b LSGDFFSYAD REDHYWTGYY TSRPFYKSLD RVLEAHLRGA EVLYSLAAAH ARRSGLAGRY PLSDFTLLTE
Hs GMIIx_v1b LSGDFFSYAD REDHYWTGYY TSRPFYKSLD RVLEAHLRGA EVLYSLAAAH ARRSGLAGRY PLSDFTLLTE
Hs GMIIx_v3b LSGDFFSYAD REDHYWTGYY TSRPFYKSLD RVLEAHLRGA EVLYSLAAAH ARRSGLAGRY PLSDFTLLTE
Hs GMII LSGDFFTYAD RDDHYWSGYF TSRPFYKRMD RIMESHLRAA EILYYFALRQ AHKYKINKFL SSSLYTALTE
561
Hs GMIIx_v1a ARRTLGLFQH HDAITGTAKE AVVVDYGVRL LRSLVNLKQV IIHAAHYLVL GDKETYHFDP EAPFLQVDDT
Hs GMIIx_v2b ARRTLGLFQH HDAITGTAKE AVVVDYGVRL LRSLVNLKQV IIHAAHYLVL GDKETYHFDP EAPFLQVDDT
Hs GMIIx_v1b ARRTLGLFQH HDAITGTAKE AVVVDYGVRL LRSLVNLKQV IIHAAHYLVL GDKETYHFDP EAPFLQVDDT
Hs GMIIx_v3b ARRTLGLFQH HDAITGTAKE AVVVDYGVRL LRSLVNLKQV IIHAAHYLVL GDKETYHFDP EAPFLQVDDT
Hs GMII ARRNLGLFQH HDAITGTAKD WVVVDYGTRL FHSLMVLEKI IGNSAFLLIG KDKLTYDSYS PDTFLEMDLK
631
Hs GMIIx_v1a RLSHDALPER TVIQLDSSPR FVVLFNPLEQ ERFSMVSLLV NSPRVRVLSE EGQPLAVQIS AHWSSATEAV
Hs GMIIx_v2b RLSHDALPER TVIQLDSSPR FVVLFNPLEQ ERFSMVSLLV NSPRVRVLSE EGQPLAVQIS AHWSSATEAV
Hs GMIIx_v1b RLSHDALPER TVIQLDSSPR FVVLFNPLEQ ERFSMVSLLV NSPRVRVLSE EGQPLAVQIS AHWSSATEAV
Hs GMIIx_v3b RLSHDALPER TVIQLDSSPR FVVLFNPLEQ ERFSMVSLLV NSPRVRVLSE EGQPLAVQIS AHWSSATEAV
Hs GMII QKSQDSLPQK NIIRLSAEPR YLVVYNPLEQ DRISLVSVYV SSPTVQVFSA SGKPVEVQVS AVWDTANTIS
701
Hs GMIIx_v1a PDVYQVSVPV RVPALGLGVL QLQLGLDGHR TLPSSVRIYL HGRQLSVSRH EAFPLRVIDS GTSDFALSNR
Hs GMIIx_v2b PDVYQVSVPV RVPALGLGVL QLQLGLDGHR TLPSSVRIYL HGRQLSVSRH EAFPLRVIDS GTSDFALSNR
Hs GMIIx_v1b PDVYQVSVPV RLPALGLGVL QLQLGLDGHR TLPSSVRIYL HGRQLSVSRH EAFPLRVIDS GTSDFALSNR
Hs GMIIx_v3b PDVYQVSVPV RLPALGLGVL QLQLGLDGHR TLPSSVRIYL HGRQLSVSRH EAFPLRVIDS GTSDFALSNR
Hs GMII ETAYEISFRA HIPPLGLKVY KILESASSNS HLADYV-LYK N----KVEDS GIFTIKNMIN TEEGITLENS


771
Hs GMIIx_v1a YMQVWFSGLT GLLKSIRRVD EEHEQQVDMQ VLVYGTRTSK DKSGAYLFLP DGEAKPYVPK EPPVLRVTEG
Hs GMIIx_v2b YMQVWFSGLT GLLKSIRRVD EEHEQQVDMQ VLVYGTRTSK DKSGAYLFLP DGEAKPYVPK EPPVLRVTEG
Hs GMIIx_v1b YMQVWFSGLT GLLKGSGLCF LAEHPKGG-- ---------- ---------- ---------- ----------
Hs GMIIx_v3b YMQVWFSGLT GLLKSIRRVD EEHEQQVDMQ VLVYGTRTSK DKSGAYLFLP DGEASPTSPR SPPCCVSLK-
Hs GMII FVLLRFD-QT GLMKQMMTKE DGKHHEVNVQ FSWYGTTIKR DKSGAYLFLP DGNAKPYVYT TPPFVRVTHG
841
Hs GMIIx_v1a PFFSEVVAYY EHIHQAVRLY NLPGVEGLSL DISSLVDIRD YVNKELALHI HTDIDSQGIF FTDLNGFQVQ
Hs GMIIx_v2b PFFSEVVAYY EHIHQAVRLY NLPGVEGLSL DISSLVDIRD YVNKELALHI HTDIDSQGIF FTDLNGFQVQ
Hs GMIIx_v1b ---------- ---------- ---------- ---------- ---------- ---------- ----------
Hs GMIIx_v3b --ALSSQRWL RTMSTFTRRS GFTICQGWRG CLWTYHPWWT SGTTSTRSWP CTSIQT---- ----STARVQ
Hs GMII RIYSEVTCFF DHVTHRVRLY HIQGIEGQSV EVSNIVDIRK VYNREIAMKI SSDIKSQNRF YTDLNGYQIQ
911
Hs GMIIx_v1a PRRYLKKLPL QANFYPMPVM AYIQDAQKRL TLHTAQALGV SSLKDGQLEV ILDRRLMQDD NRGLGQGLKD
Hs GMIIx_v2b PRRYLKKLPL QANFYPMPVM AYIQDAQKRL TLHTAQALGV SSLKDGQLEV ILDRRLMQDD NRGLGQGLKD
Hs GMIIx_v1b ---------- ---------- ---------- ---------- ---------- ---------- ----------
Hs GMIIx_v3b PRRYLKKLPL QANFYPMPVM AYIQDAQKRL TLHTAQALGV SSLKDGQLEV ILDRRLMQDD NRGLGQGLKD
Hs GMII PRMTLSKLPL QANVYPMTTM AYIQDAKHRL TLLSAQSLGV SSLNSGQIEV IMDRRLMQDD NRGLEQGIQD
981
Hs GMIIx_v1a NKRTCNRFRL LLERRTVGS- ---------- ---------- ----EVQDSH STSYPSLLSH LTSMYLNAPA
Hs GMIIx_v2b NKRTCNRFRL LLERRTVGSE PDFFSKLAAM FRGLIFHSSR SGNREVQDSH STSYPSLLSH LTSMYLNAPA
Hs GMIIx_v1b ---------- ---------- ---------- ---------- ---------- ---------- ----------
Hs GMIIx_v3b NKRTCNRFRL LLERRTVGS- ---------- ---------- ----EVQDSH STSYPSLLSH LTSMYLNAPA
Hs GMII NKITANLFRI LLEKRSAVN- ---------- ---------- ----TEEEKK SVSYPSLLSH ITSSLMNHPV
1051
Hs GMIIx_v1a LALPVARMQL PGPGLRSFHP LASSMPCDFH LLNLRTLQAE EDTLPSAETA LILHRKGFDC GLEAKNLGFN
Hs GMIIx_v2b LALPVARMQL PGPGLRSFHP LASSMPCDFH LLNLRTLQAE EDTLPSAETA LILHRKGFDC GLEAKNLGFN
Hs GMIIx_v1b ---------- ---------- ---------- ---------- ---------- ---------- ----------
Hs GMIIx_v3b LALPVARMQL PGPGLRSFHP LASSLPCDFH LLNLRTLQAE EDTLPSAETA LILHRKGFDC GLEAKNLGFN
Hs GMII IPMANKFSSP TLELQGEFSP LQSSLPCDIH LVNLRTIQSK VGNGHSNEAA LILHRKGFDC RFSSKGTGLF
1121
Hs GMIIx_v1a CTTSQGKVAL GSLFHGLDVV FLQPTSLTLL YPLASPSNST DVYLEPMEIA TFRLRLG
Hs GMIIx_v2b CTTSQGKVAL GSLFHGLDVV FLQPTSLTLL YPLASPSNST DVYLEPMEIA TFRLRLG
Hs GMIIx_v1b ---------- ---------- ---------- ---------- ---------- -------
Hs GMIIx_v3b CTTSQGKVAL GSLFHGLDVV FLQPTSLTLL YPLASPSNST DVYLEPMEIA TFRLRLG
Hs GMII CSTTQGKILV QKLLNKFIVE SLTPSSLSLM HSPPGTQNIS EINLSPMEIS TFRIQLR

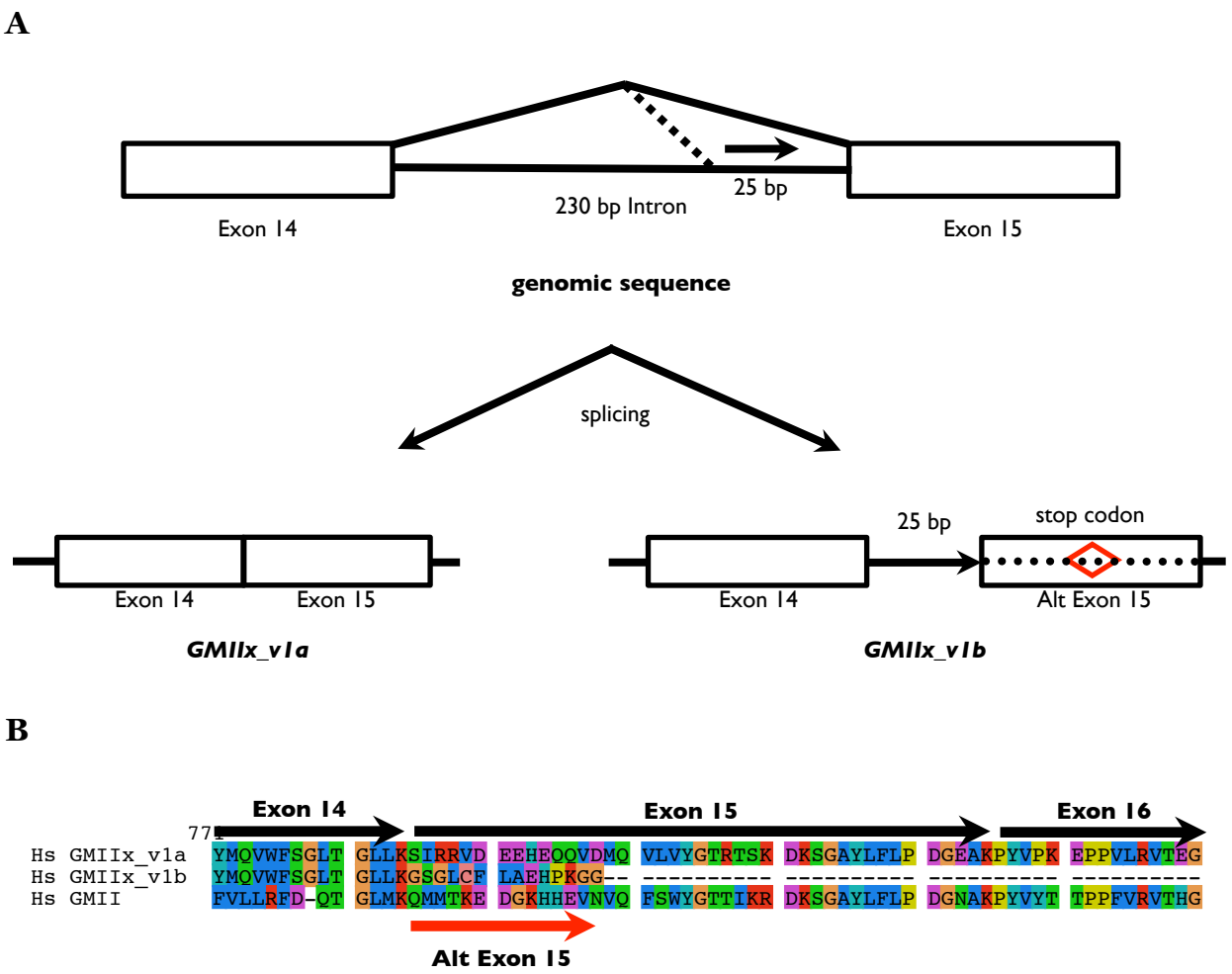
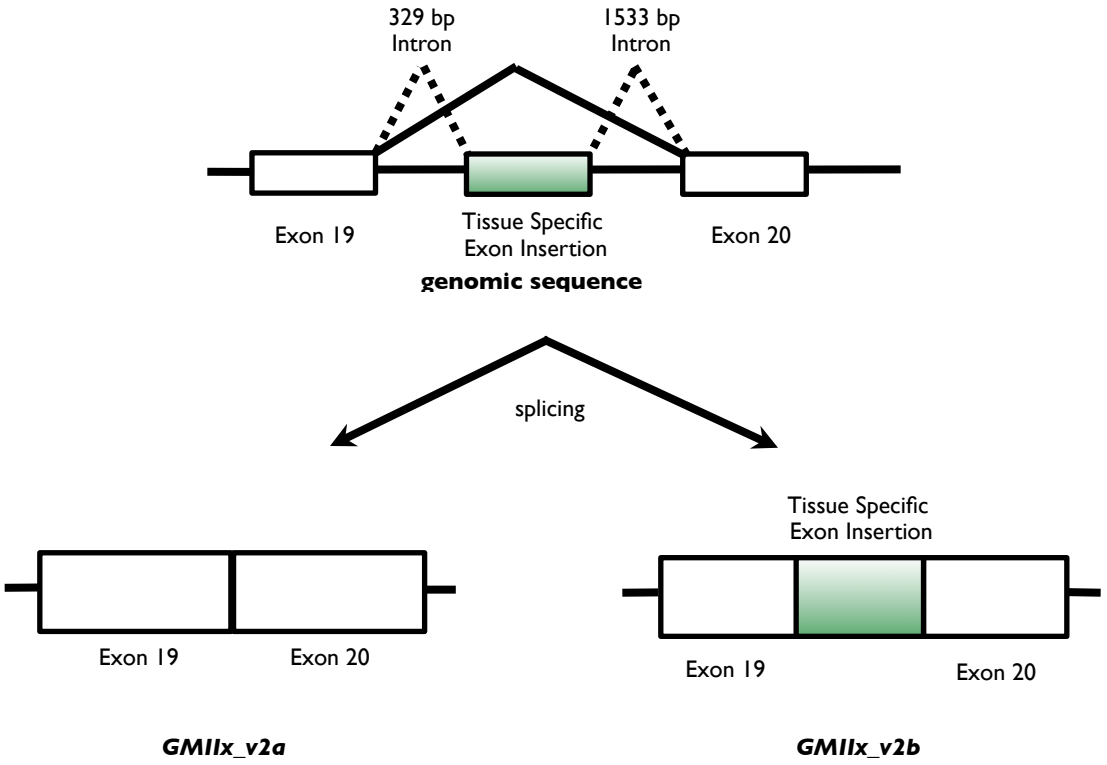


Fig. 2.3. GMIIx is alternatively spliced in melanoma cells. (A) This figure shows that GMIIx is normally transcribed as exon 14 and exon 15 in the full length protein as GMIIx_v1a. In melanoma cells, 25 bp of intronic sequence is transcribed leading to the expression of alternative exon 15 leading to a frameshift in the reading frame and the expression of a premature stop codon. As a result, translation of the GMIIx_v1b mRNA leads to the expression of a truncated protein. (B) Protein sequence alignment of GMIIx splice isoforms GMIIx_v1a and GMIIx_v1b with human GMII. The red arrow depicts the expression of alternative exon 15 leading to the synthesis of a truncated polypeptide. Misago, et al. (1995). *Proc Natl Acad Sci U S A* **92**(25):11766-11770.

A



B

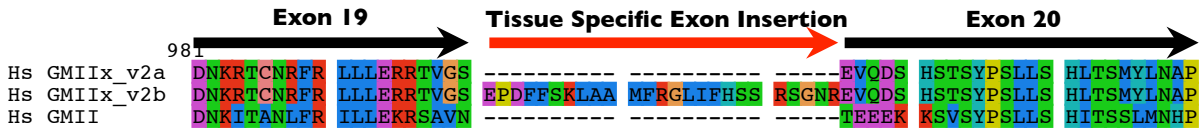
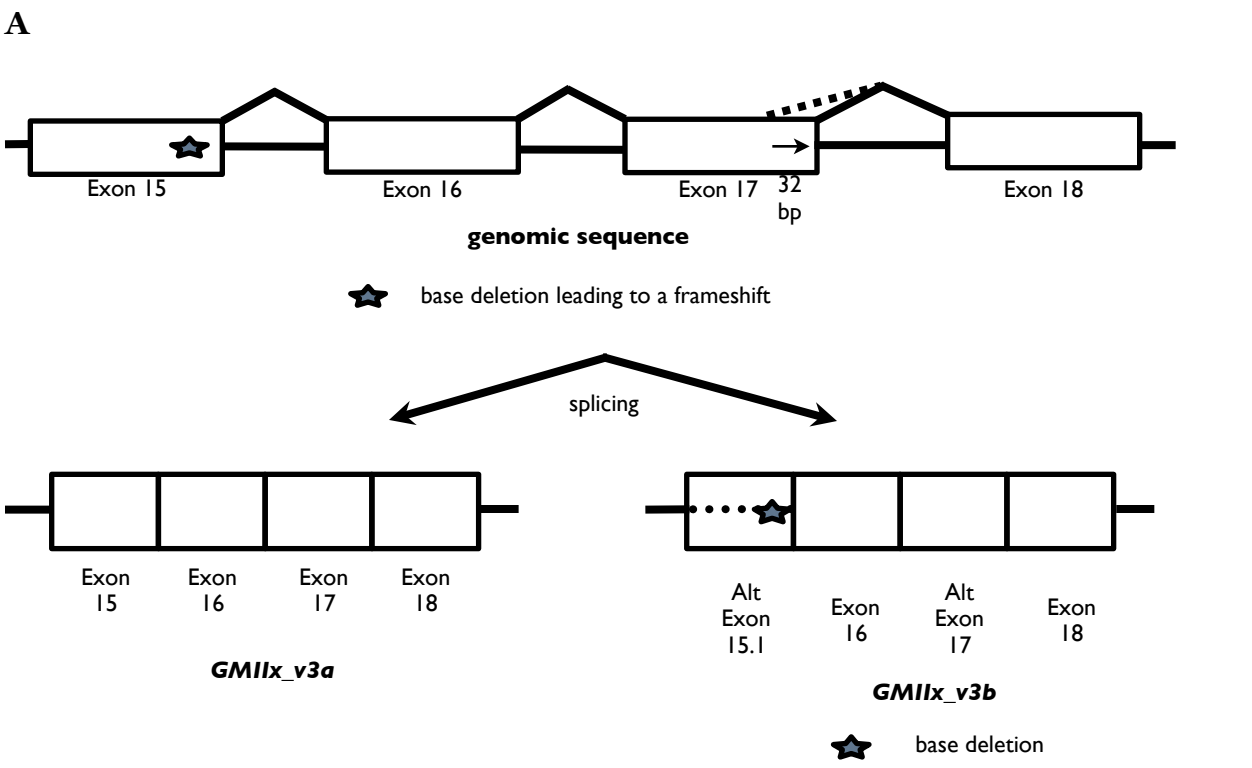


Fig. 2.4. GMIIx is expressed as a tissue specific isoform in human brain tissue. (A) Transcription of exon 19 and exon 20 leads to the expression of GMIIx_v2a. In human brain tissue, alternative splicing generates a transcript with a tissue specific exon inseration following exon 19 leading to the expression of GMIIx_v2b. (B) Sequence alignment of GMIIx splice isoforms, GMIIx_v2a and GMIIx_v2b with human GMII. The red arrow depicts the tissue specific exon insertion in human brain tissue leading to the translation of an additional 25 aa.

Misago, et al. (1995). *Proc Natl Acad Sci U S A* **92**(25):11766-11770.



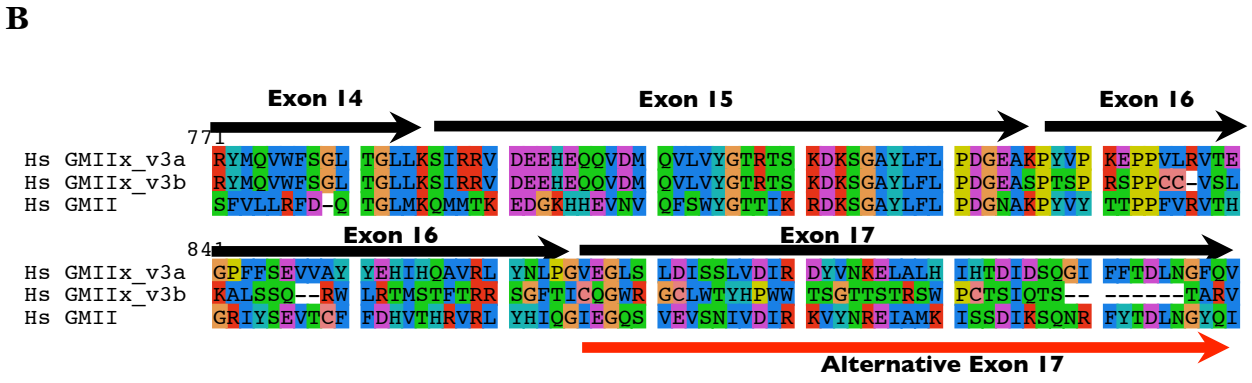
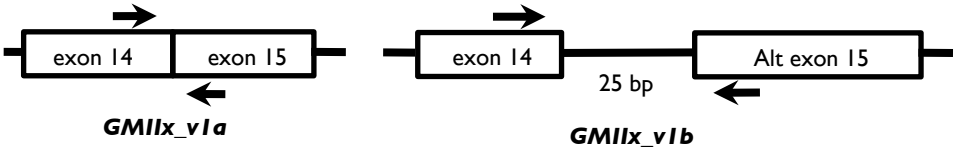


Fig. 2.5. GMIIx is alternatively spliced in melanoma cells. (A) GMIIx_v3a is transcribed as exon 15, exon 16, exon 17 and exon 18. In melanoma cells, a base pair deletion in the region transcribed by exon 15 leads to a frameshift in reading frame. Expression of alternative exon 17 results in a deletion of 32 bp and the restoration of the reading frame in GMIIx_v3b. (B) Protein sequence alignment of GMIIx_v3a and GMIIx_v3b with human GMII. The red arrow represents alternative exon 17. Misago, et al. (1995). *Proc Natl Acad Sci U S A* **92**(25):11766-11770.

A



B

GMIIx splice variant	Product size
GMIIx_v1a	75 bp
GMIIx_v1b	100 bp

C

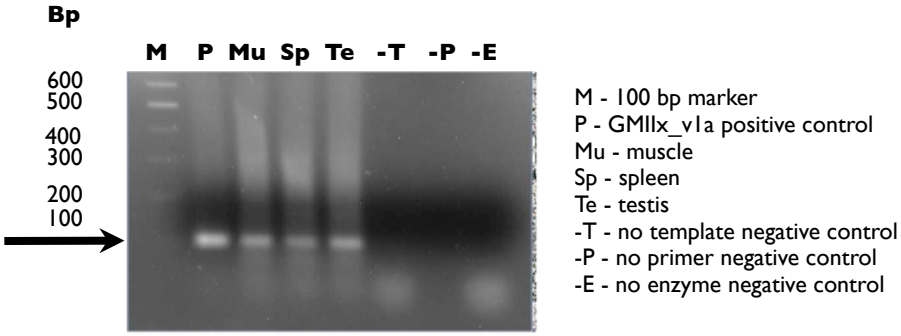
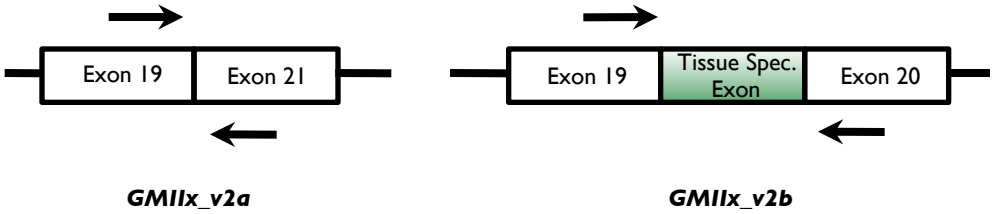


Fig. 2.6. GMIIx_v1a is expressed in human muscle, spleen and testis as determined by RT-PCR. (A) Location of the gene specific primers for RT-PCR in GMIIx_v1a and GMIIx_v1b transcripts. (B) Predicted sizes of the PCR products for the GMIIx splice isoforms. (C) GMIIx transcript expression was examined in human muscle, spleen and testis tissues by RT-PCR. The black arrow shows the expression of a 75 bp PCR product corresponding to the expression of GMIIx_v1a. The expression of a 100 bp PCR product corresponding to the expression of GMIIx_v1b was not detected.

A



B

GMIIx splice variant	Product size
GMIIx_v2a	109 bp
GMIIx3_v2b	184 bp

C

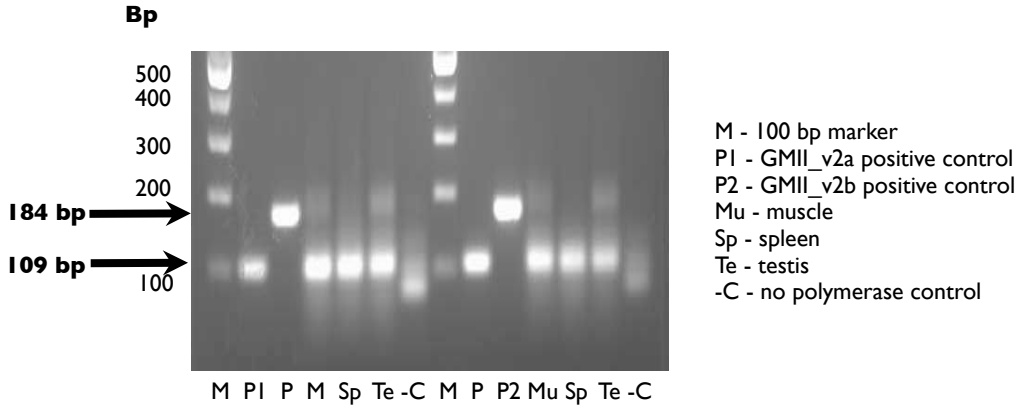


Fig. 2.7. GMIIx_v2a is expressed in human muscle, spleen and testis as determined by RT-PCR. (A) Location of the gene specific primers for RT-PCR in GMIIx_v2a and GMIIx_v2b transcripts. (B) Predicted sizes of PCR products for the GMIIx splice isoforms. (C) GMIIx transcript expression in human muscle, spleen and testis tissues was examined by RT-PCR. The black arrows show the corresponding sizes of the PCR products. The top arrow represents a PCR product of 184 bp corresponding to expression of GMIIx_v2b. The bottom arrow represents a PCR product of 109 bp corresponding to the expression of GMIIx_v2a

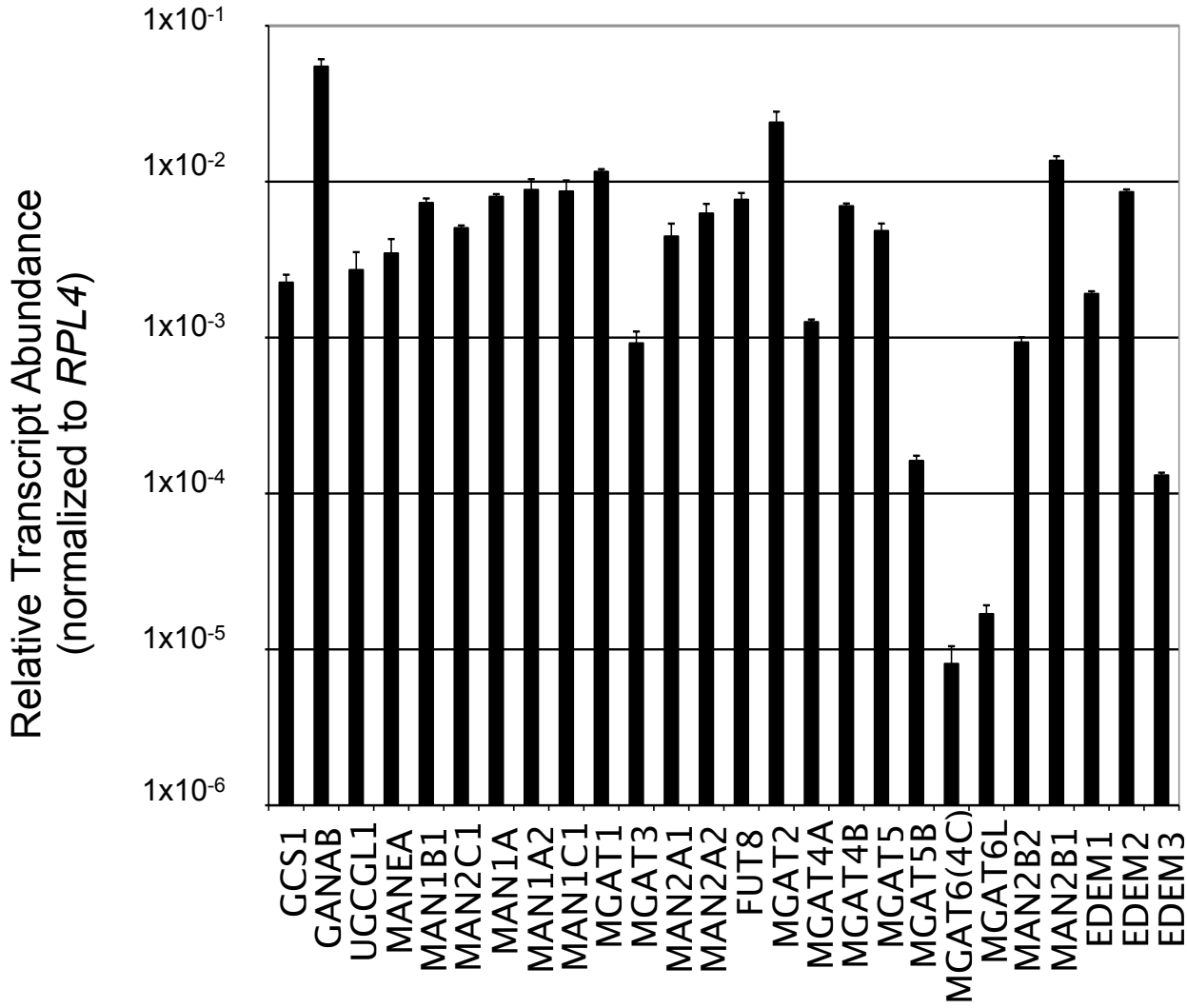


Fig. 2.8. mRNA transcript expression of genes contributing to N-glycan biosynthesis or catabolism was quantitated in mouse livers by qRT-PCR. The relative transcript abundance was normalized to the expression levels of ribosomal protein L4 (RPL4). Nairn, et al. (2008). *J Biol Chem* **283**(25):17298-17313.

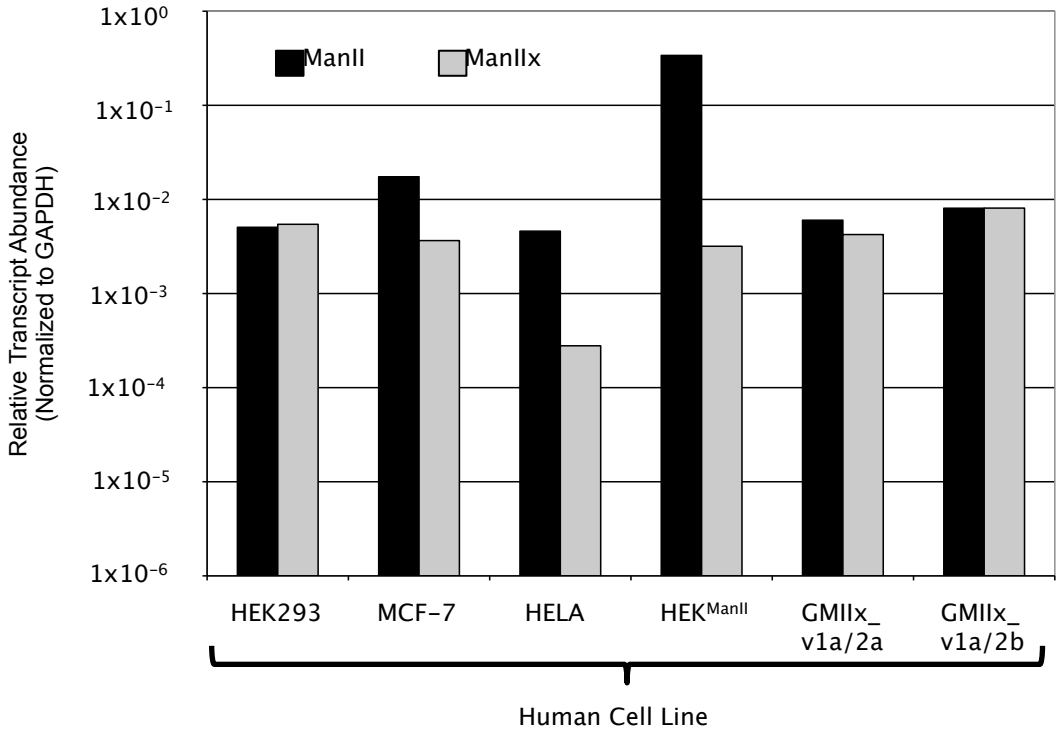


Fig. 2.9. GMII and GMIIx transcript expression in various mammalian cell lines was quantitated by qRT-PCR in HEK293 cells, MCF-7 breast cancer cells, HeLa cells, HEK293 cells transfected with GMII, HEK293 cells transfected with GMIIx_v1a/2a, and HEK293 cells transfected with GMIIx_v1a/2b. Relative transcript abundance was normalized to the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

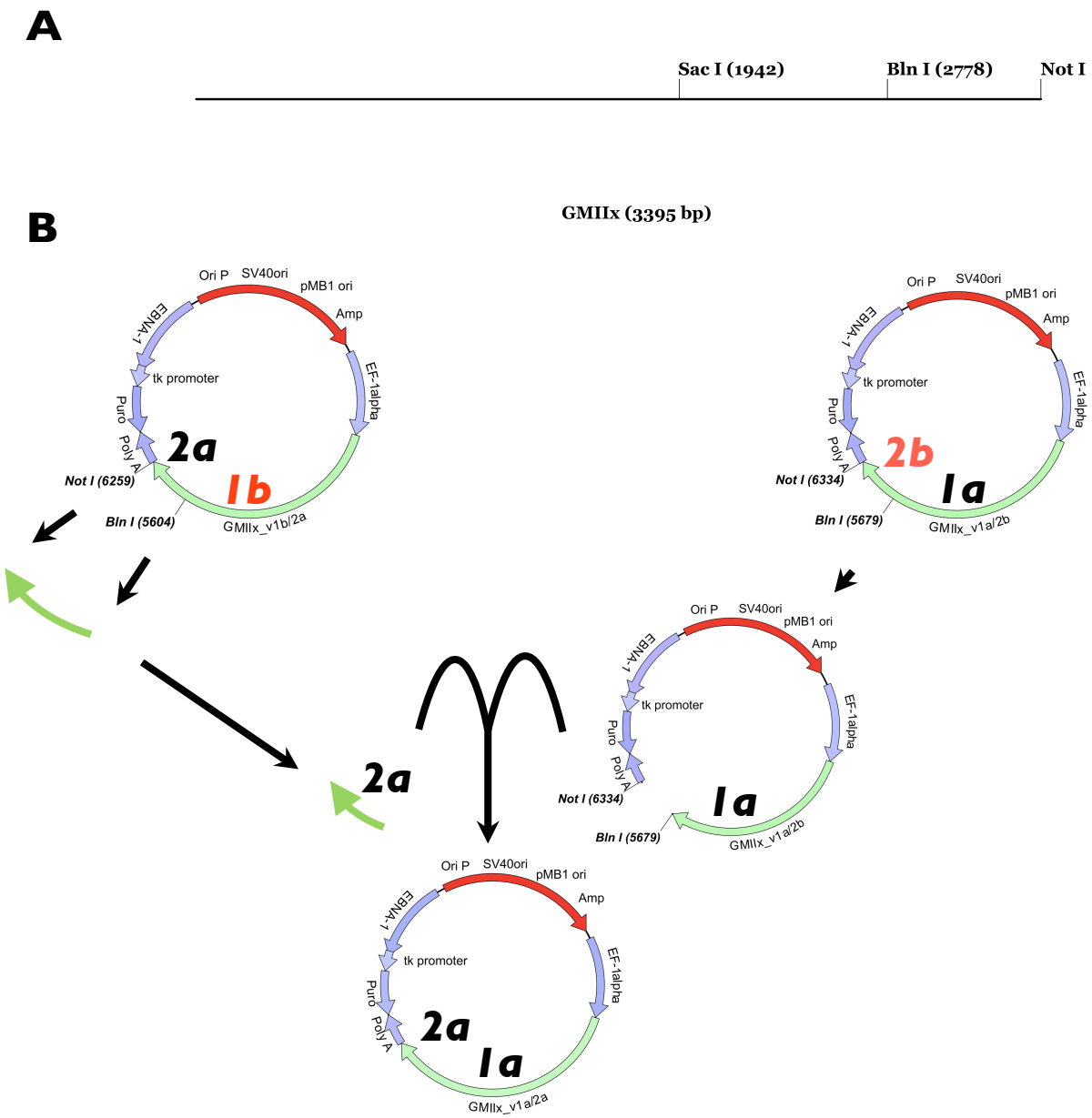


Fig. 2.10. An expression vector for the soluble catalytic domain of GMIIx with greatest similarity to class II mannosidase was constructed. (A) A restriction map of the GMIIx_v1a mRNA showing the locations of the Sac I, Bln I and Not I restriction sites. A region from the 3' end of GMIIx_v1b/2a spanning the Bln I - Not I restriction sites was excised and swapped into the corresponding region in GMIIx_v1a/2b to generate GMIIx_v1a/2a.

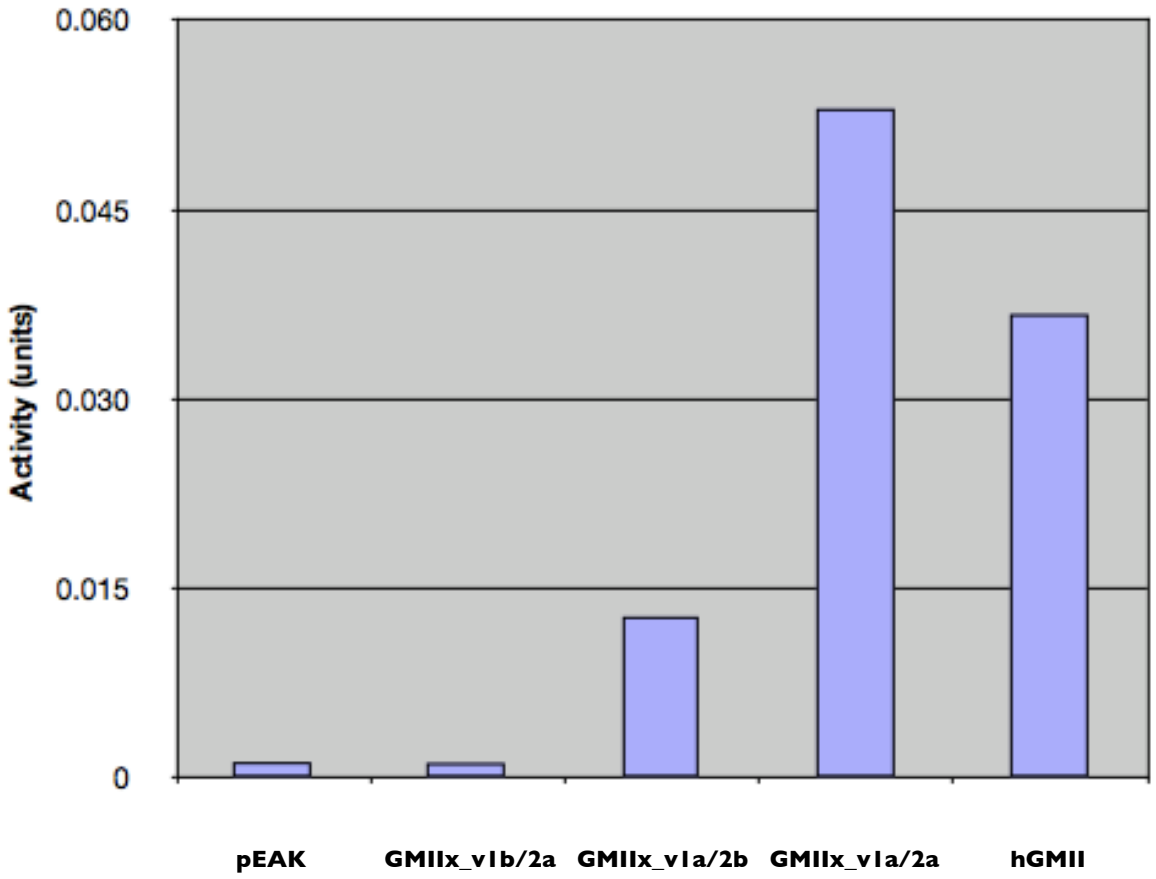


Fig. 2.11. The proteins translated by GMIIx splice isoforms display differential levels of α-mannosidase activity. GMIIx splice isoforms GMIIx_v1b/2a, GMIIx_v1a/2b, GMIIx_v1a/2a and recombinant hGMII were transfected into HEK293 cells. Conditioned media from transfected HEK293 cells was assayed with 4-MU α-Man for 1 hour at 37°C and pH 5.6. PEAK: pEAK10 expression vector, GMIIx_v1b/2a: a human melanoma cDNA clone (GenBank L28821), GMIIx_v1a/2b: a human brain cDNA clone (GenBank BI757742), GMIIx_v1a/2a: recombinant α-mannosidase IIx protein based on similarity to Class II mannosidases, GMII: human Golgi α-mannosidase II (GenBank NM_002372).

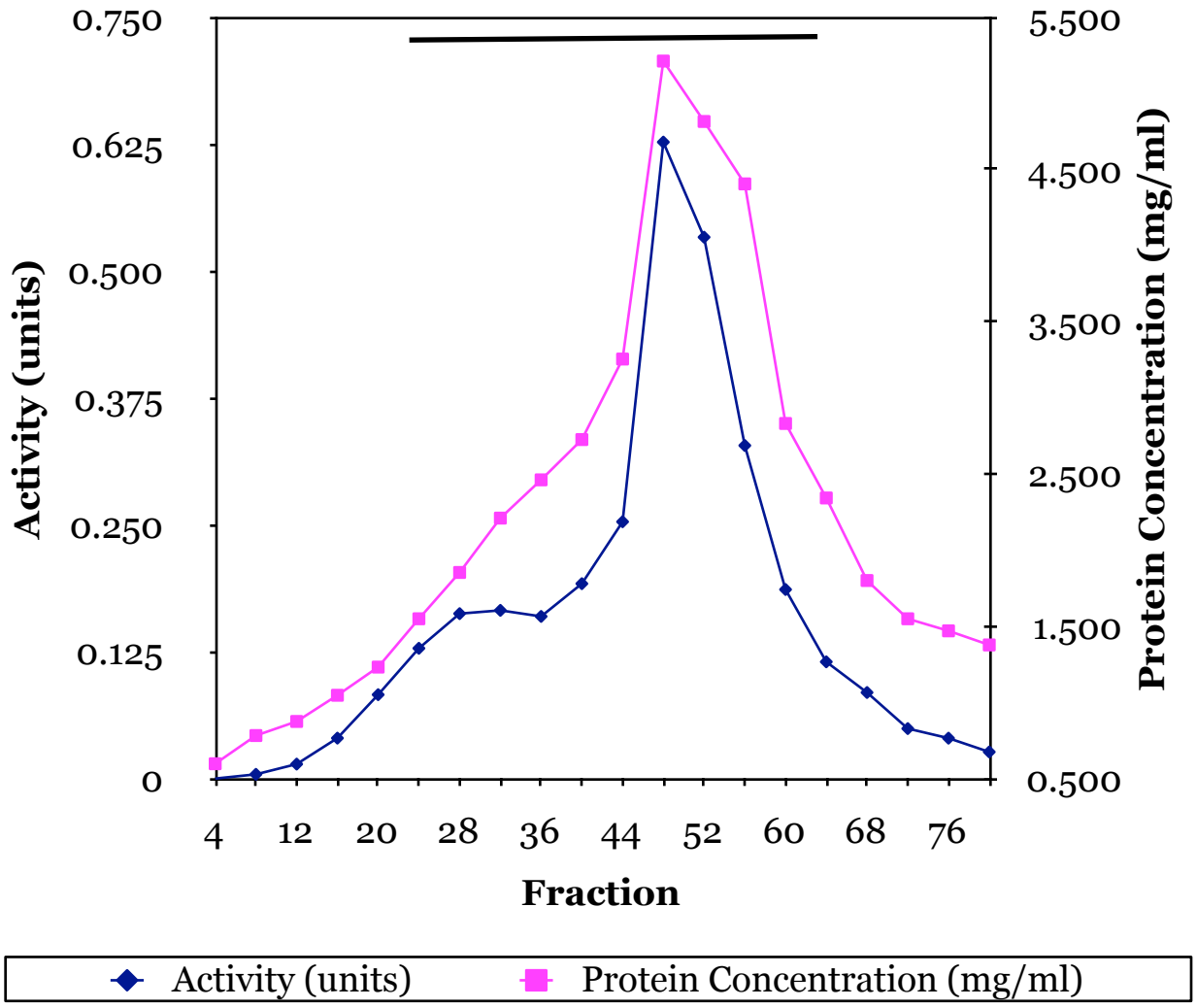


Fig. 2.12. Phenyl Sepharose chromatography of human recombinant Golgi α-mannosidase IIx. Ammonium sulfate was added to conditioned media from rGMIIx stably transfected HEK293 cells to a final concentration of 400 mM. The media was loaded onto a phenyl sepharose column pre-equilibrated in buffer A. rGMIIx was eluted with a 0-100% gradient of 50% ethylene glycol. Fractions were collected and assayed for protein concentration and enzyme activity. The black bar represents fractions that were pooled for further purification.

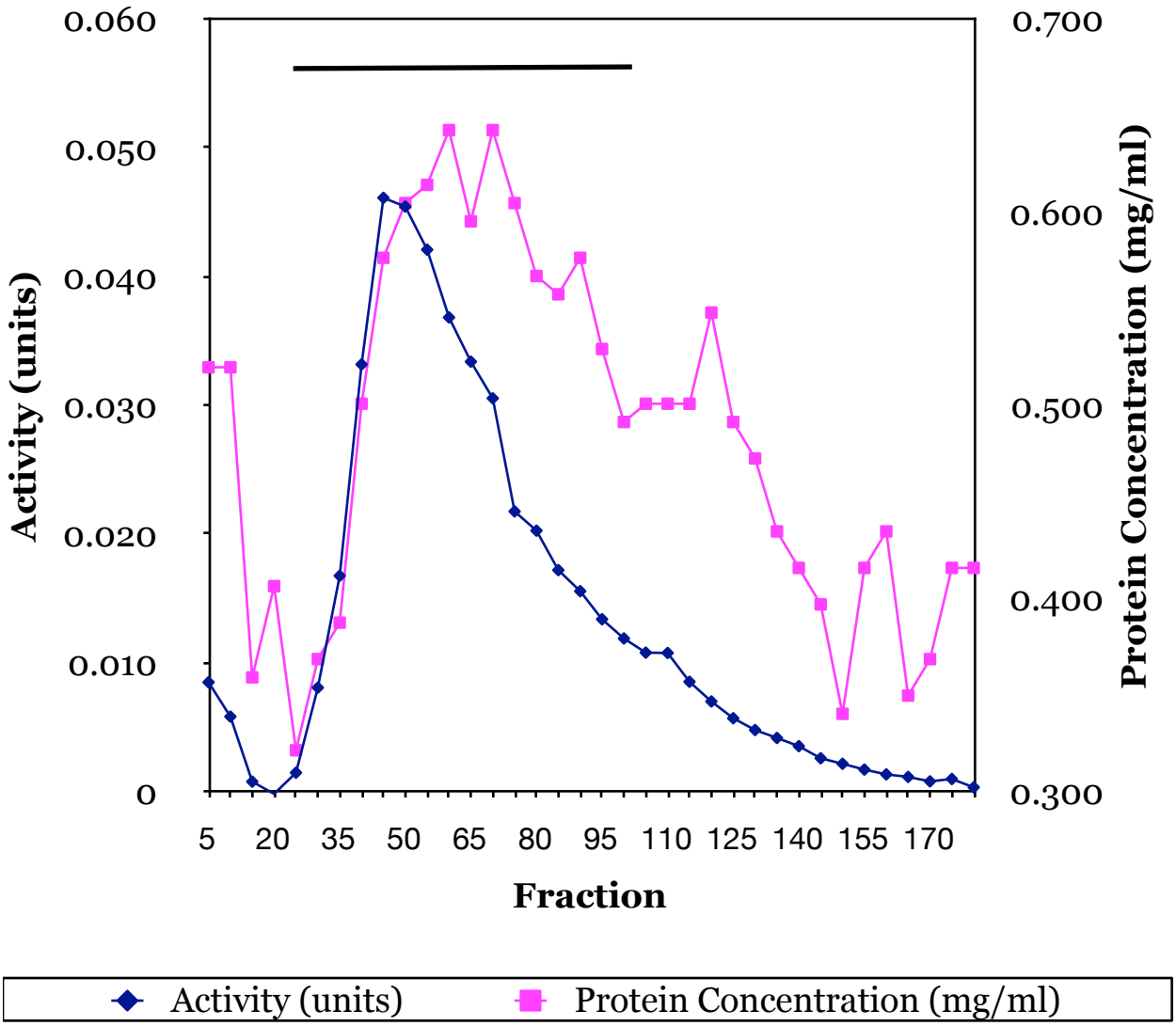


Fig. 2.13. Blue Sepharose chromatography of human recombinant Golgi α-mannosidase IIx. The pool from the phenyl sepharose chromatography step was dialyzed and loaded onto a pre-equilibrated blue sepharose column. rGMIIx was eluted with a 0-100% gradient of 1 M sodium chloride. Fractions were collected and assayed for protein concentration and enzyme activity. The black bar represents fractions that were pooled for further purification.

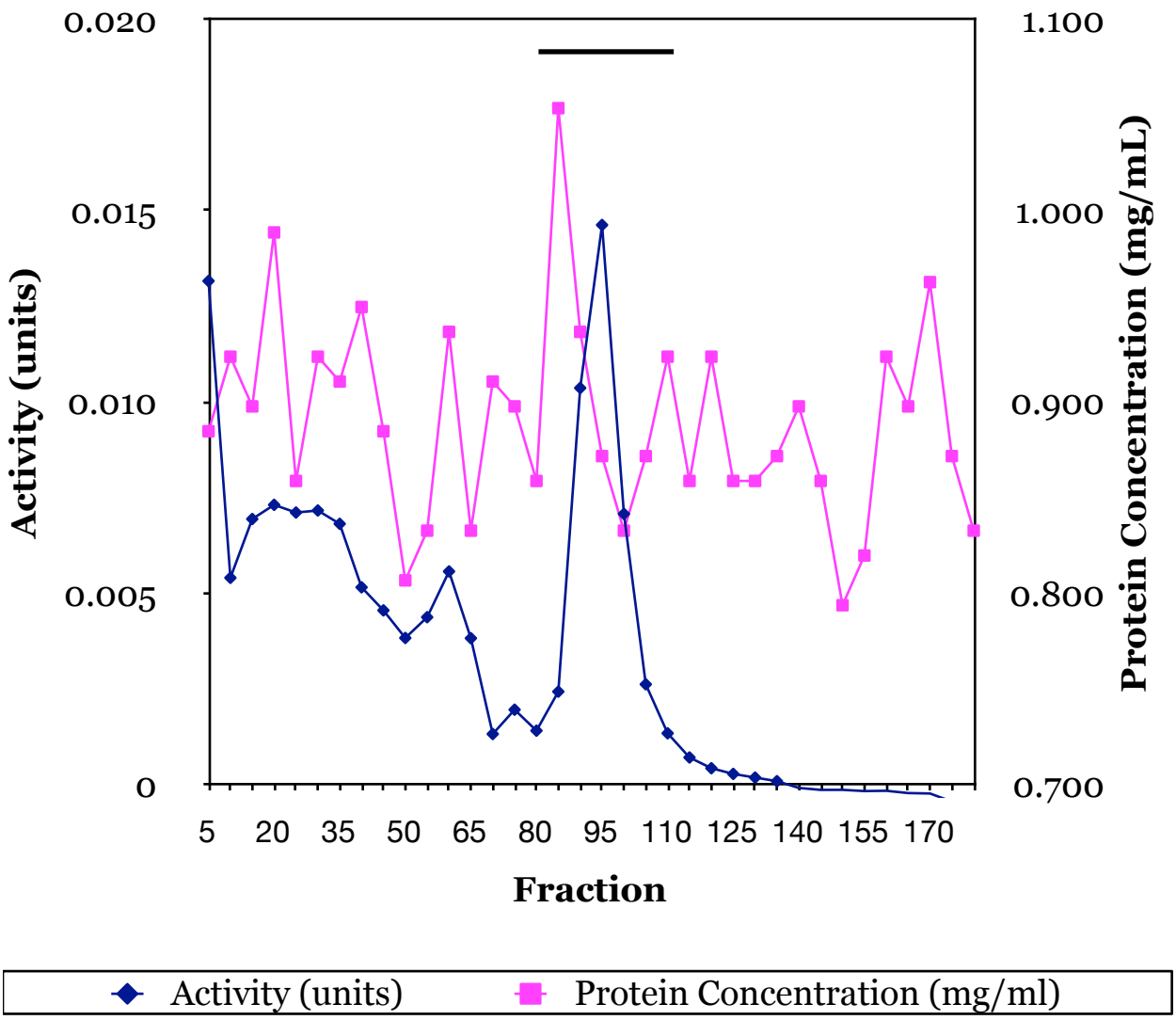


Fig. 2.14. Hydroxyapatite chromatography of human recombinant Golgi α-mannosidase IIx. The pool from the phenyl sepharose chromatography step was diluted and loaded onto a pre-equilibrated hydroxyapatite column. rGMIIx was eluted with a 0-100% gradient of 200 mM phosphate buffer, pH 7.5. Fractions were collected and assayed for protein concentration and enzyme activity. The marked fluctuations in protein concentration likely represent background noise. The black bar represents fractions that were pooled for characterization.

Table 2.1. Purification of recombinant human Golgi α-mannosidase IIx. Conditioned media was harvested and processed with the indicated chromatographies. Protein concentration was measured using the BCA assay and enzyme activity was tracked using a 4-MU α-Man assay.

Fraction	Total Protein	Activity	Recovery	Specific Activity	Fold Purification
	(mg)	(units)	%	(units/mg)	
Conditioned Media	5310.2	12.0	100.0	0.0	-
Phenyl Sepharose	345.1	4.9	40.6	0.1	51.9
Blue Sepharose[a]	72.8	1.4	28.7	0.5	225.4
CHT I [b]	8.3	0.1	2.0	1.2	506.9

a Blue sepharose 6L CB chromatography

b CHT I hydroxyapatite chromatography

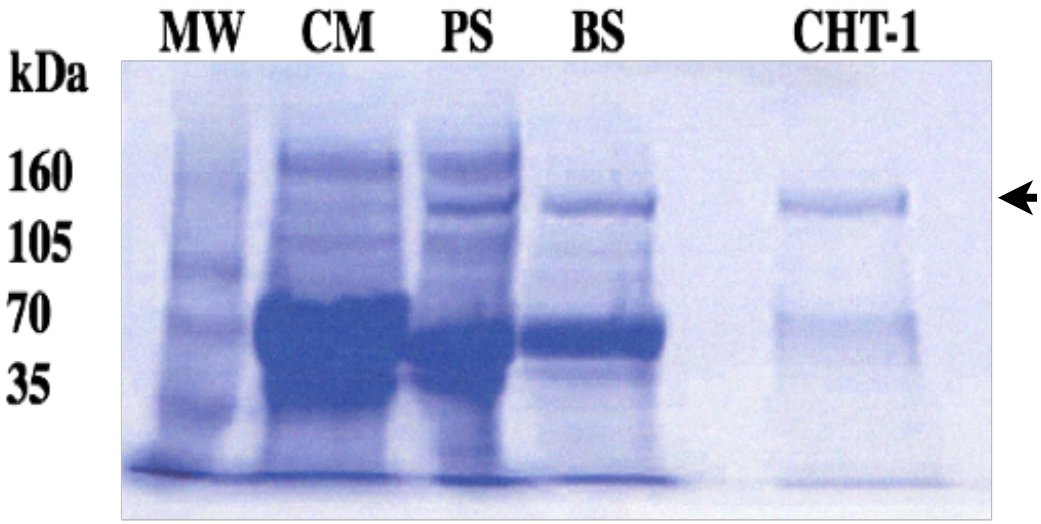


Fig. 2.15. SDS-PAGE of rGMIIx. Samples were electrophoresed on an 8% acrylamide gel. The band migrating at 130 kDa is consistent with the size of GMIIx and the band at migrating at 60 kDa is most likely represents residual serum albumin from the conditioned media. CM: conditioned media, PS: phenyl sepharose purified pool, BS: blue sepharose purified pool, CHT-1: hydroxyapatite purified pool. Arrowhead indicates the enriched protein preparation that was confirmed by sequencing by mass spectrometry.

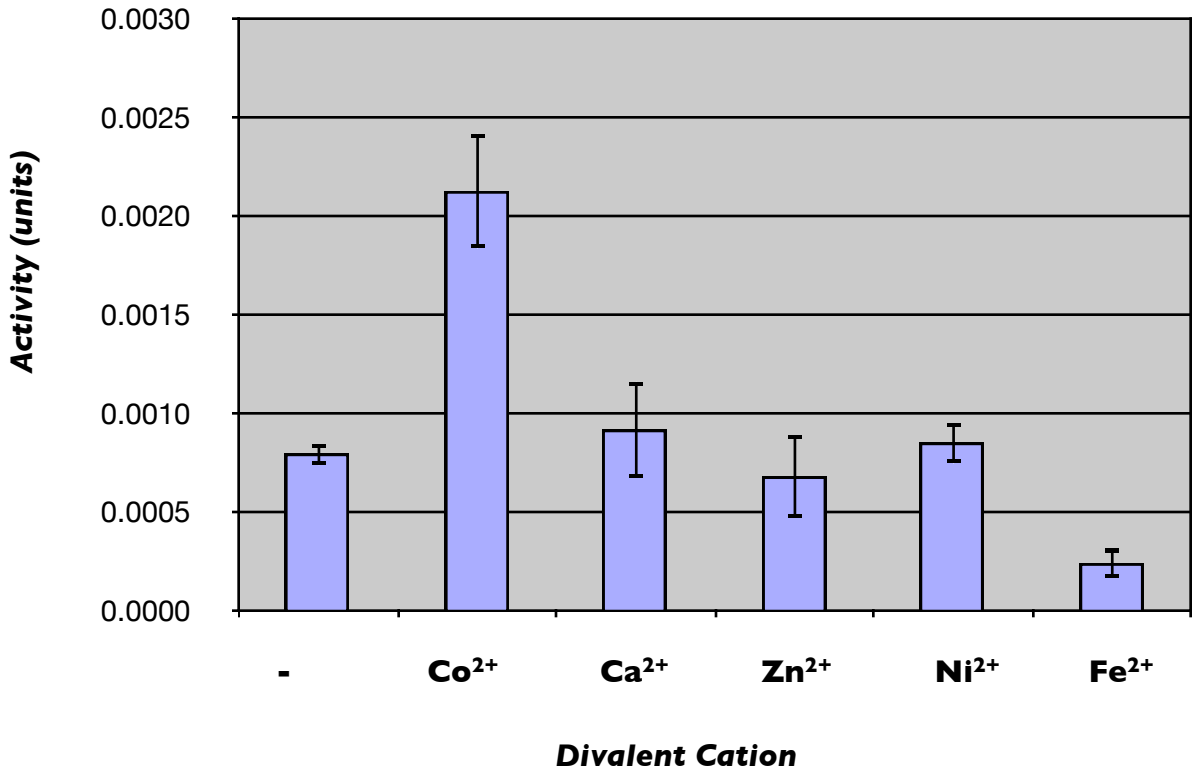


Fig. 2.16. rGMIIx is stimulated 2.5 fold in the presence of Co^{2+} and is inhibited 3 fold by Fe^{2+}. GMIIx was assayed with 4-MU α-Man at 37°C for 1 hour and pH 5.6. The enzyme was assayed alone or in the presence of 5 mM Co^{2+}, 5 mM Ca^{2+}, 5 mM Zn^{2+} , 5 mM Ni^{2+} or 5 mM Fe^{2+}.

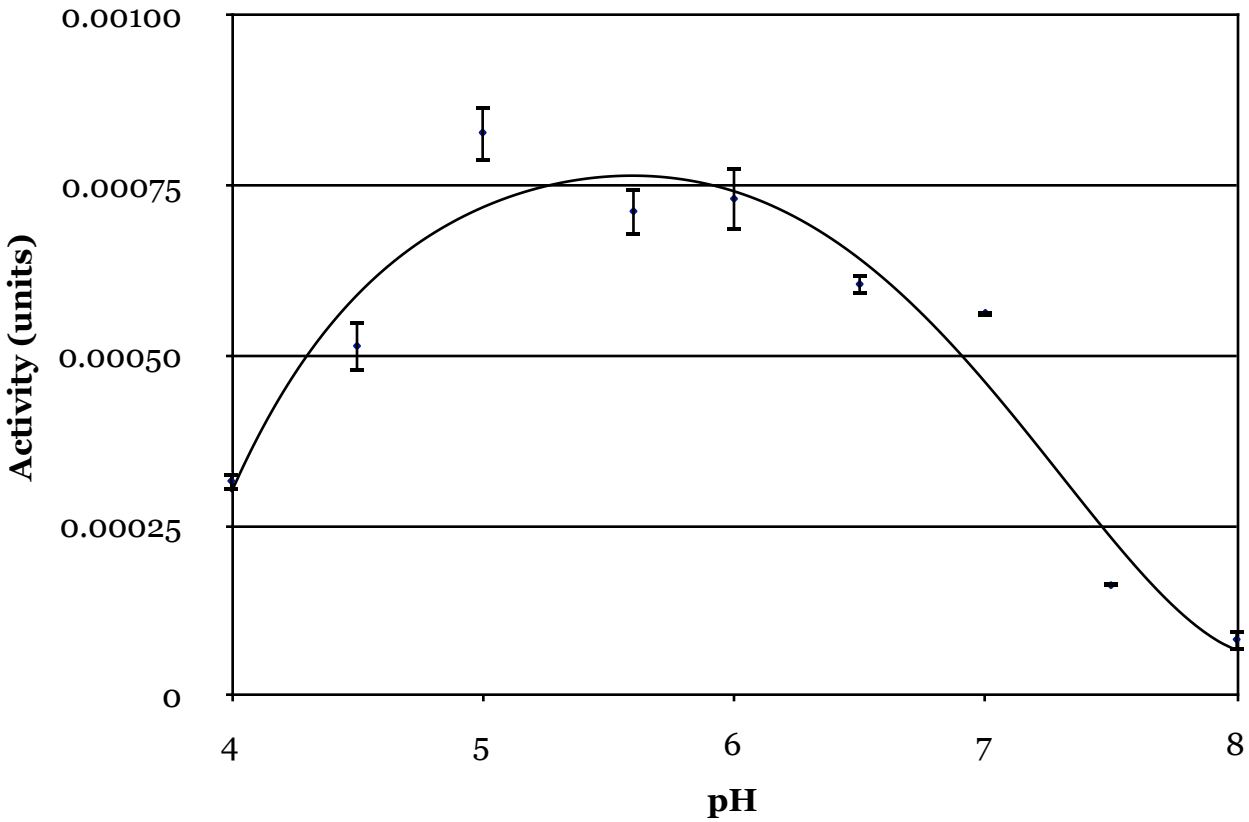


Fig. 2.17. pH dependence of rGMIIx activity. rGMIIx was assayed with 4-MU α-Man at 37°C for 1 hour in buffers over a pH range of 4.0 to 8.0. The enzyme has an optimum of activity at pH 5.6.

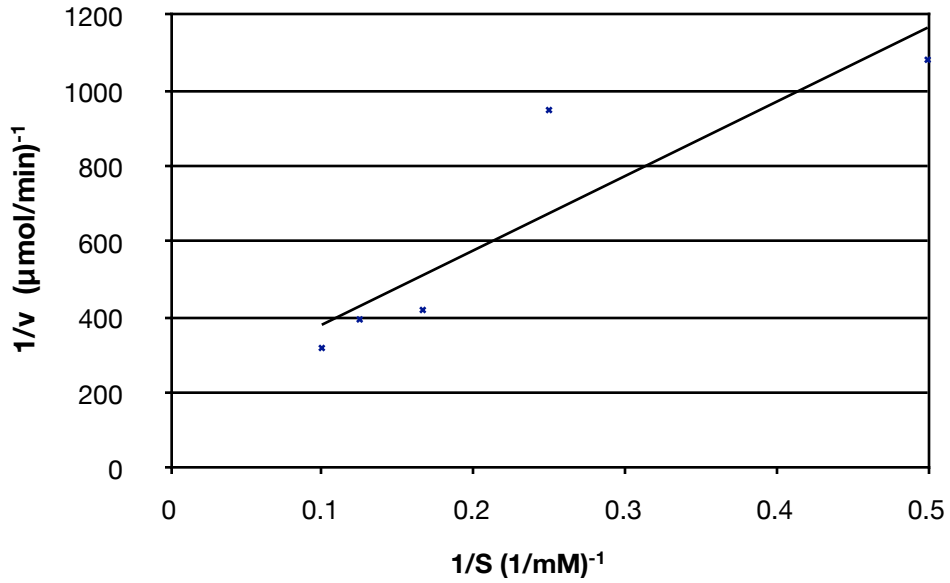


Fig. 2.18. Lineweaver-Burk plot of rGMIIx activity. rGMIIx was assayed with increasing concentrations of 4-MU α-Man for 1 hour at pH 5.6.

Table 2.2. Kinetic data for human recombinant Golgi α-mannosidase IIx. The Michaelis-Menten kinetic constants K_m and V_{max} were determined from the linear regression of the data points obtained by assaying rGMIIx with 4-MU α-Man.

	Kinetic Constants	
	K_m (mM)	v_{max} (nmol/min)
GMIIx	10.7	5.4

Table 2.3. Comparison of known K_m values for α-mannosidases involved in the biosynthesis or catabolism of N-glycoproteins.

	Substrate	K_m (mM)	References
Rn GMII	pNP-Man	40	(Moremen, et al., 1991)
Mm LysMan	pNP-Man	12.6	(Merkle, et al., 1997)
Hs LysMan	pNP-Man	2.4	(Liao, et al., 1996)
Hs α1,6 LysMan	4-MU α-Man	7.6	(Park, et al., 2005)
Hs ERMI	$Man_9GlcNAc$	0.4	(Trembley and Herscovics, 1999)
Mm GMIA	Manα1,2Manα-O-CH_3	1.5	(Lal, et al., 1998)
Mm GMIB	Manα1,2Manα-O-CH_3	2	(Lal, et al., 1998)
Sc α1,2 Mannosidase	$Man_9GlcNAc$	0.3	(Lipari and Herscovics, 1994)

Table 2.4. Inhibitory constants for human recombinant Golgi α-mannosidase IIx. (A) Inhibitory constants for DIM and Sw upon GMII. (B) Inhibitory constants for DIM and Sw upon rGMIIx. (C) The inhibitors kifunensine and deoxymannojirimycin have IC_{50} values against rGMIIx in the millimolar range. GMII or GMIIx was assayed with increasing concentrations of 4-MU α-Man at varying inhibitor concentrations. The inhibitory constants were obtained by replotting the data as described previously (Segel, 1993).

A

GMII	Inhibitory Constants
Inhibitor	Ki (μM)
DIM	3.5
Swainsonine	0.04

B

GMIIx	Inhibitory Constants
Inhibitor	Ki (μM)
DIM	416.0
Swainsonine	0.2

C

GMIIx	
Inhibitor	IC_{50} (mM)
Kifunensine	1.1
DMJ	1.2

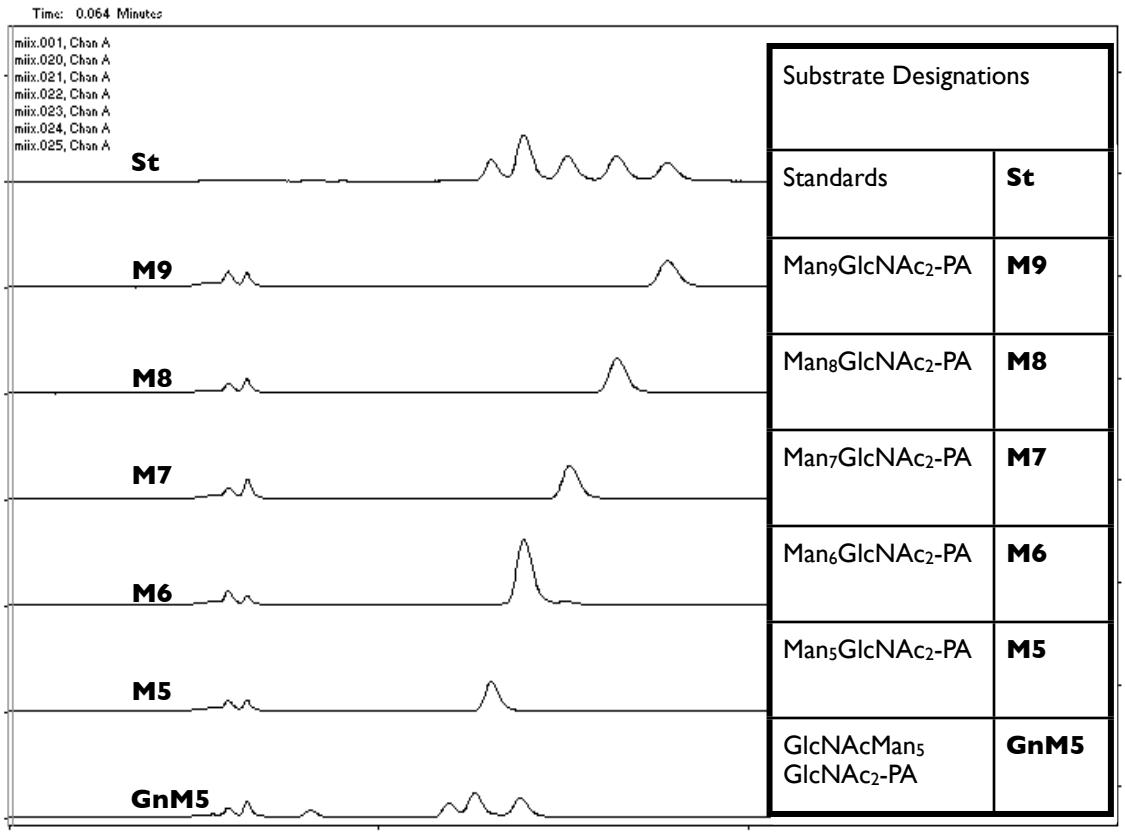


Fig. 2.19. rGMIIx is able to cleave $GlcNAcMan_5GlcNAc_2$-PA, but is unable to cleave high mannose oligosaccharide substrates. GMIIx was incubated with 10 nmol of oligosaccharide at pH 5.6 for 24 hours at 37°C. Product formation was analyzed by HPLC. The oligosaccharide substrates employed in the assays were: 1) $Man_9GlcNAc_2$-PA, 2) $Man_8GlcNAc_2$-PA, 3) $Man_7GlcNAc_2$-PA, 4) $Man_6GlcNAc_2$-PA, 5) $Man_5GlcNAc_2$-PA, and 6) $GlcNAcMan_5GlcNAc_2$-PA. The identity of the peaks was confirmed by coelution with a pool of undigested oligosaccharide substrates, $Man_{9\text{-}5}GlcNAc_2$-PA, representing a standard (St).

Table 2.5. Substrate specificity study of GMII in wild type mouse liver. Cell fractions were prepared from wild-type mouse liver and immunoprecipitated with an anti rat Golgi α-mannosidase II antibody. Immunocomplexes were assayed with PA-tagged oligosaccharide substrates and product formation was determined by HPLC.

Wt Mouse Liver	**Cell Fraction**		
	Homogenate	**Cytosol**	**Microsomal/ Golgi Membranes**
Substrate	**Cleavage (Product)**	**Cleavage (Product)**	**Cleavage (Product)**
M5Gn2	no	no	no
M5Gn	no	no	no
GnM5Gn2	yes (GnM3Gn2)	yes (GnM3Gn2)	yes (GnM3Gn2)
GnM5Gn	yes (GnM3Gn)	no	yes (GnM3Gn)

Table 2.6. Substrate specificity study of GMIIx in wild type mouse liver. Cell fractions were prepared from wild type mouse liver and immunoprecipitated with an anti-human Golgi α-mannosidase IIx antibody. Immunocomplexes were assayed with PA-tagged oligosaccharide substrates and product formation was determined by HPLC.

Wt Mouse Liver	**Cell Fraction**		
	Homogenate	**Cytosol**	**Microsomal/ Golgi Membranes**
Substrate	**Cleavage (Product)**	**Cleavage (Product)**	**Cleavage (Product)**
M5Gn2	no	no	no
M5Gn	no	no	no
GnM5Gn2	no	no	no
GnM5Gn	no	no	no

Table 2.7. Substrate specificity study of CytMan in wild type mouse liver. Cell fractions were prepared from wild type mouse liver and immunoprecipitated with an anti-rat cytosolic α-mannosidase antibody. Immunocomplexes were assayed with PA-tagged oligosaccharide substrates and product formation was determined by HPLC.

Wt Mouse Liver	**Cell Fraction**		
	Homogenate	**Cytosol**	**Microsomal/ Golgi Membranes**
Substrate	**Cleavage (Product)**	**Cleavage (Product)**	**Cleavage (Product)**
M5Gn2	very minor	no	no
M5Gn	yes (M3Gn)	yes (M3Gn)	yes (M4Gn)
GnM5Gn2	very minor (GnM4Gn2)	no	no
GnM5Gn	yes (GnM4Gn)	yes (GnM4Gn)	no

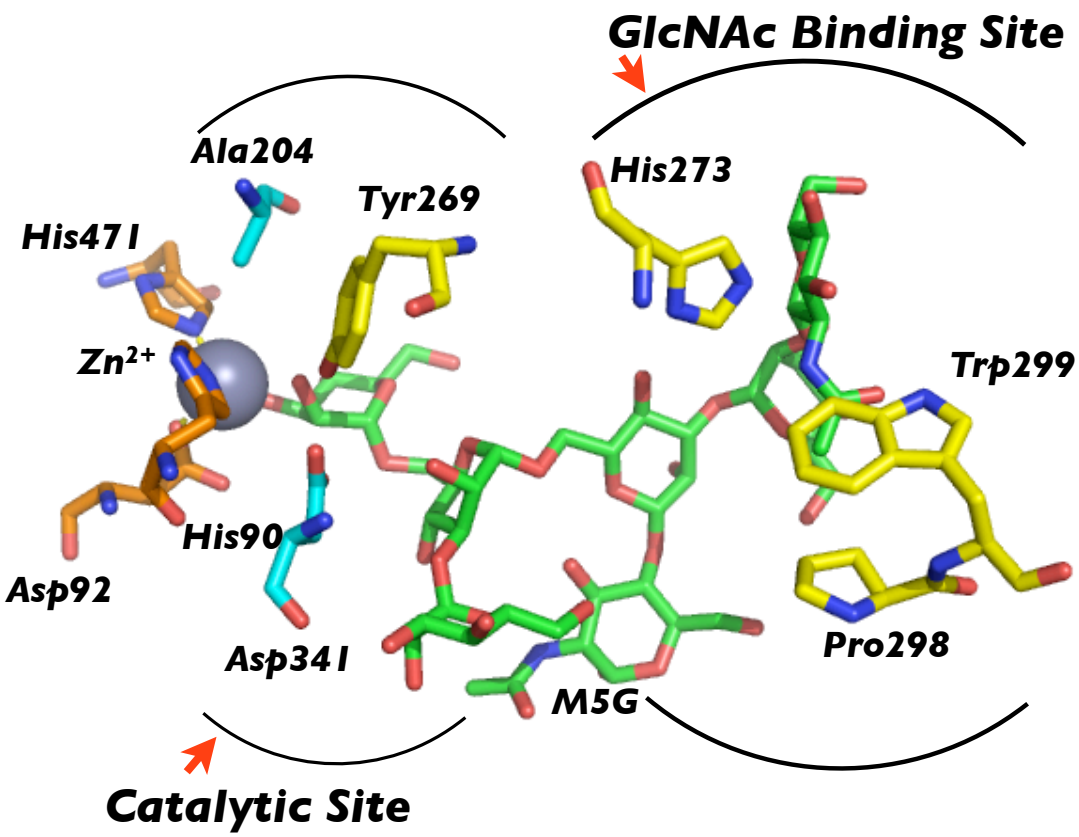


Fig. 2.20. Structure of the *Drosophila melanogaster* Golgi α-mannosidase II active site bound with the oligosaccharide substrate $GlcNAcMan_5GlcNAc$ (M5G). A mutant protein was crystallized with the conserved catalytic residue D204 mutated to alanine. The catalytic site containing the conserved amino acid residues D341 and Y269 and the GlcNAc binding site containing the conserved amino acid residues H273, P298 and W299 are indicated.

Shah, N., et al. (2008). *Proc. Natl. Acad. Sci. U. S. A.* **105**:9570-9575.

Chapter 3

DISCUSSION

The biosynthesis of complex N-glycans is important for normal development and physiology. Alterations in the N-glycan biosynthetic pathway lead to the incidence of pathological states including cancer, congenital disorders of glycosylation (CDGs), and diabetes. In this work, it is shown that human Golgi α-mannosidase II and human Golgi α-mannosidase IIx are isozymes responsible for the conversion of hybrid type oligosaccharides to complex type oligosaccharides by catalyzing functionally redundant pathways.

Based on previous attempts to characterize the substrate specificity of human GMIIx, it was unclear whether GMIIx hydrolyzes high mannose type oligosaccharides. Protein sequence alignment of GMIIx with GMII indicates that the region containing the active site residues is highly conserved between the two isozymes. It suggested the idea that GMIIx is able to hydrolyze the α1→6 and α1→3 terminal mannose residues from the hybrid type oligosaccharide $GlcNAcMan_5GlcNAc_2$ to produce $GlcNAcMan_3GlcNAc_2$ based on the substrate specificity of GMII (Tulsiani, et al., 1982). This contradicts indirect biochemical evidence from transfection of full length human GMIIx into CHO mammalian cells leading to a reduction of the high mannose oligosaccharide $Man_6GlcNAc_2$ and an accumulation of $Man_4GlcNAc_2$ (Oh-Eda, et al.,

2001). It should be noted that while GMIIx activity against the synthetic substrates pNP-Man and 4-MU α-Man was detected, the activity levels were extremely low, did not require any divalent cation, and were unable to hydrolyze natural oligosaccharide substrates (Misago, et al., 1995, Oh-Eda, et al., 2001). The inconsistency between the structural data and the biochemical data was the driving force behind the need for more detailed information about human GMIIx.

Two models have been proposed to explain GMIIx-mediated processing of N-linked oligosaccharides. The first model was proposed based on the analysis of the GMII null mouse. Germline disruption of GMII in the mouse leads to a dyserythropoietic anemia similar to HEMPAS where cells from the erythoid lineage express reduced levels of complex N-glycans (Chui, et al., 1997). This suggested that another mannosidase activity was present in most tissues compensating for the lack of GMII enzyme (Chui, et al., 1997). Additionally, another mannosidase activity was discovered that hydrolyzed $Man_5GlcNAc$-PA in the presence of cobalt termed GMIII (Chui, et al., 1997). Based on these results, it was concluded that GMIIx is a likely candidate enzyme able to compensate for the loss of GMII activity and catalyze an alternative pathway to form complex type N-glycans that bypass the GMII step.

The hypothesis of an alternative pathway executed by a "GMIII" activity is difficult to characterize, since only trace activity levels were detected in GMII null animal tissues (Chui, et al., 1997). Based on the substrate specificity of the activity detected in these tissues, GMIII is believed to be identical to a mannosidase activity originally identified by Bonay and Hughes that cleaves α 1→6, α 1→3, and α 1→2 linkages in a cobalt dependent manner in rat liver (Bonay and Hughes, 1991, Oh-Eda, *et*

al., 2001, Akama, et al., 2006). While it is possible that the alternative pathway could be catalyzed by a homolog of GMII, similar to the substrate specificity of Dm α-mannosidase IIb and *Spodoptera frugiperda* Golgi α-mannosidase III, an enzyme with this specificity has not been experimentally isolated from mammalian sources (Francis, et al., 2002, Strachan, 2009). GMIIx was implicated in the pathway based on indirect evidence from characterization of a GMIIx transfected CHO cell line employing radiolabeled oligosaccharides, and ConA chromatography of N-glycan structures showing that the levels of $Man_6GlcNAc_2$ are reduced and the levels of $Man_4GlcNAc_2$ are elevated (Oh-Eda, et al., 2001). This suggested that GMIIx was catalyzing the processing of high mannose oligosaccharides. Our data confirm the existence of the alternative pathway by showing that an activity immunoprecipitated from mouse liver microsomal/ Golgi membranes by the anti-cytosolic α-mannosidase antibody is able to cleave $Man_5GlcNAc$-PA in a cobalt dependent manner. However, GMIIx protein was not detected in any untransfected cell line or mouse tissue indicating that the activity hydrolyzing $Man_5GlcNAc$-PA in mouse liver microsomal/Golgi membranes is not GMIIx.

With the emergence of detailed structural information of the catalytic mechanism of GMII, inferences about the substrate specificity of GMIIx can be drawn. Based on structural analysis of inhibitors and the oligosaccharides $GlcNAcMan_5GlcNAc_2$ and $Man_5GlcNAc_2$ bound to the active site of Dm GMII, three groups of amino acid residues were identified based on their function. R228, D472 and R876 are inhibitor stablizing residues, Y267, D204 and D341 are the conserved catalytic nucleophile and catalytic acid/base, and W299, P298 and H273 are residues necessary for GlcNAc

binding and subsequent catalysis (Fig. 3.1) (Shah, et al., 2008). The GlcNAc binding residues are essential for selective catalysis of the substrate GlcNAc $Man_5GlcNAc_2$ whereas high mannose oligosaccharides such as $Man_5GlcNAc_2$ are able to bind weakly to the active site but are not cleaved (Shah, et al., 2008). Coupled with assay data from the Dm GMII enzyme, where GMII hydrolyzed $GlcNAcMan_5GlcNAc_2$ with an 80 fold preference over $Man_5GlcNAc_2$, it seems unlikely that GMIIx is directly hydrolyzing high mannose oligosaccharides in the alternative pathway (Zhong, et al., 2008).

The second model proposed for GMIIx function was based on studies performed in animal models and mammalian cell culture. Disruption of the *Man2a2* gene in the mouse led to generation of a GMIIx null mouse (Akama, et al., 2002). Characterization of the GMIIx null mouse showed that males are infertile with reduced testes and defective spermatogenesis due to aberrant complex type oligosaccharide production leading to a failure in germ cell-Sertoli cell adhesion (Akama, et al., 2002). It remained unclear whether GMIIx was catalyzing a GMII redundant pathway based on these data. Generation of a double null GMII/GMIIx double null mouse resulted in mice that died shortly after birth due to respiratory failure with abnormal vacuoles and enlarged mitochondria in the lungs and liver (Akama, et al., 2006). Complex type N-linked oligosaccharide production was abrogated in the absence of both enzymes and complex type N-glycan production could be restored in double null fibroblasts by transfection with either GMII or GMIIx (Akama, et al., 2006). Characterization of glycan expression in GMII and GMIIx null embryos showed that GMIIx null embryos produce complex oligosaccharides similar to wild type embryos, GMII null embryos express elevated levels of hybrid type oligosaccharides and reduced levels of complex type

oligosaccharides, and GMII/GMIIx double null embryos do not express any detectable complex type oligosaccharides (Akama, et al., 2006).

Concurrently, when a recombinant murine GMIIx catalytic domain was transfected into COS cells, GMIIx hydrolyzed the hybrid type oligosaccharide $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_3GlcNAc_2$-PA; however, the high mannose type oligosaccharides $Man_6GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA were not cleaved (Akama, et al., 2006). These data confirm that GMIIx and GMII catalyze a functionally redundant pathway in a tissue-specific manner and GMIIx is responsible for producing complex type oligosaccharides in the absence of GMII in the GMII null mouse. This work shows that a recombinant human GMIIx catalytic domain transfected into HEK293 cells hydrolyzes the hybrid type oligosaccharide $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_3GlcNAc_2$-PA, but is unable to cleave high mannose oligosaccharides, consistent with the data from the mouse variant of the enzyme. Collectively, these data show that in mammals, GMIIx is responsible for generation of complex type N-glycans.

It is interesting to note the distinct evolution of two apparently redundant enzyme systems in the Golgi apparatus in mammals versus insect and potentially other non-mammalian organisms. In insects, one GH38 enzyme isoform retains the specificity of mammalian GMII, requiring the prior addition of a GlcNAc by GNTI. Despite sharing the three Pfam regions, Glyco_hydro 38, alpha-mann mid, and Glyco_hydro 38C, the other isoform is able to cleave $Man_5GlcNAc_2$ structures directly to $Man_3GlcNAc_2$ without GNTI action allowing a potential alternative bypass pathway. The implication is that a GMIIx ortholog may be involved in hydrolysis of high mannose oligosaccharides in

lower eukaryotes, but, in mammals, GMIIx has lost the ability to process such oligosaccharides and retains an enzyme specificity similar to GMII. Insights into the evolution and biological role of GMIIx based on further biochemical characterization will be important for understanding the roles that these enzymes play in alternative processing pathways in different organisms.

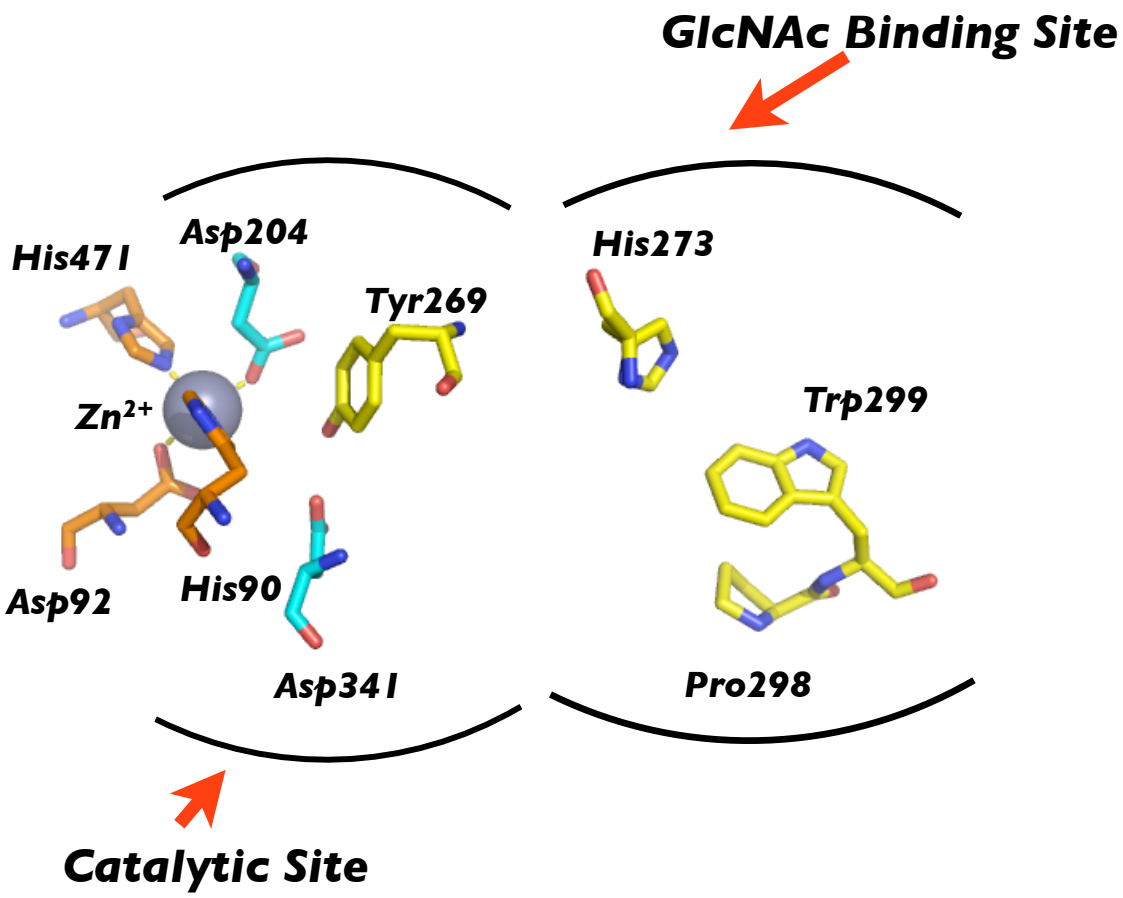


Fig. 3.1. Structure of the *Drosophila melanogaster* Golgi α-mannosidase II active site . The catalytic site containing the conserved amino acid residues D204, D341 and Y269 and the GlcNAc binding site containing the conserved amino acid residues H273, P298 and W299 are indicated.

van den Elsen, J. M., et al. (2001). *EMBO J.* **20**:3008-3017.

REFERENCES

Akama, T. O., Nakagawa, H.., Sugihara, K., Narisawa, S., Ohyama, C., Nishimura, S.-I., O'Brien, D. A., Moremen, K. W., Millan, J. L. and M. N. Fukuda. (2002). Germ cell survival through carbohydrate-mediated interaction with Sertoli cells. *Science* **295**(124):124-127.

Akama, T. O., Nakagawa, H., Wong, N. K., Sutton-Smith, M., Dell, A., Morris, H. R., Nakayama, J., Nishimura, S.-I., Pai, A., Moremen, K. W., Marth, J. D. and M. N. Fukuda. (2006). Essential and mutually compensatory role of α-mannosidase II and α-mannosidase IIx in N-glycan processing *in vivo* in mice. *Proc Natl Acad Sci U S A* **103**(24):8983-8988.

Arendt, C. W., Dawicki, W. and H. L. Ostergaard. (1999). Alternative splicing of transcripts encoding the alpha- and beta-subunits of mouse glucosidase II in T lymphocytes. *Glycobiology* **9**(3):277-283.

Berg, T., King, B., Meikle, P. J., Nilssen, O., Tollersrud, O. K. and J. J. Hopwood. (2001). Purification and characterization of recombinant human lysosomal α-mannosidase. *Mol Genet and Metab* **73**(1):18-29.

Bonay, P. and R. C. Hughes. (1991). Purification and characterization of a novel broad specificity (alpha 1----2, alpha 1----3, and alpha 1----6) mannosidase from rat liver. *Eur J Biochem* **197**(1):229-238.

Chen, L., Zhang, W., Fregien, N. and M. Pierce. (1998). The her-2/neu oncogene stimulates the transcription of N- acetylglucosaminyltransferase V and expression of its cell surface oligosaccharide products. *Oncogene* **17**(16): 2087-2093.

Chui, D., Oh-Eda, M., Liao, Y.-F., Panneerselvam, K., Lal, A., Marek, K. W., Freeze, H. H., Moremen, K. W., Fukuda, M. N. and J. D. Marth. (1997). Alpha-mannosidase II deficiency results in dyseryhropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. *Cell* **90**:157-167.

Costanzi, E., Balducci, C., Cacan, R., Duvet, S., Orlacchio, A. and T. Beccari. (2006). Cloning and expression of mouse cytosolic α-mannosidase (Man2c1). *Biochim Biophys Acta* **1760**:1580-1586.

Crispin, M., Chang, V. T., Harvey, D. J., Dwek, R. A., Evans, E. J., Stuart, D. I., Jones, E. Y., Lord, J. M., Spooner, R. A. and S. J. Davis. (2009). A human embryonic kidney 293T cell line mutated at the Golgi alpha-mannosidase II locus. *J Biol Chem* **284**(32):21684-21695.

Daniel, P. F., Winchester, B. and C. D. Warren. (1994). Mammalian alpha-mannosidases-multiple forms but a common purpose?" *Glycobiology* **4**(5):551-566.

Davies, G. and B. Henrissat. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure* **3**:853-859.

Diaz, A., Reginato, A. M. and S. A. Jimenez. (1992). Alternative splicing of human prostaglandin G/H synthase mRNA and evidence of differential regulation of the resulting transcripts by transforming growth factor beta 1, interleukin 1 beta, and tumor necrosis factor alpha. *J Biol Chem* **267**(15):10816-10822.

Francis, B. R., Paquin, L., Weinkauf, C., and D. L. Jarvis. (2002). Biosynthesis and processing of *Spodoptera frugiperda* alpha-mannosidase III. *Glycobiology* **12**(6):369-377.

Freeze, H. H. (2007). Congenital disorders of glycosylation: CDG-I, CDG-II, and beyond. *Curr. Mol. Med.* **7**:389-396.

Fukuda, M. N. (1990). HEMPAS disease: genetic defect of glycosylation. *Glycobiology* **1**(1):9-15.

Fukuda, M. N. (1993) Congenital dyserythropoietic anaemia type II (HEMPAS) and its molecular basis. *Baillieres Clin Haematol* **6**(2):493-511.

Fukuda, M. N., Papayannopoulou, T., Gordon-Smith, E. C., Rochant, H. and U. Testa. (1984). Defect in glycosylation of erythrocyte membrane proteins in congenital dyseryhtropoietic anemia type II (HEMPAS). *Br J Haematol* **56**(1):55-68.

Fukuda, M. N. (1999). HEMPAS. Hereditary erythroblastic multinuclearity with positive acidified serum lysis test. *Biochim Biophys Acta* **1455**(2-3):231-239.

Hamilton, S. R., Bobrowicz, P., Bobrowicz, B., Davidson, R. C., Li, H., Mitchell, T., Nett, J. H., Rausch, S., Stadheim, T. A., Wischnewski, H., Wildt, S., and T. U. Gerngross. (2003). Production of complex human glycoproteins in yeast. *Science* **301**(1244):1244-1246.

Hato, M., Nakagawa, H., Kurogochi, M., Akama, T. O., Marth, J. D., Fukuda, M. N. and Nishimura, S.-I. (2006). Unusual N-glycan structures in α-mannosidase II/IIx double null enbryos identified by a systematic glycomics approach based on two dimensional LC mapping and matrix-dependent selective fragmentation method in MALDI-TOF/TOF mass spectrometry. *Mol Cell Proteomics* **5**(11):2146-2157.

Heimpel, H., Anselstetter, V., Chrobak, L., Denecke, J., Einsiedler, B., Gallmeier, K., Greisshammer, A., Marquadt, T., Janka-Schaub, G., Kron, M. and E. Kohne. (2003). Congenital dyserythropoietic anemia type II: epidemiology, clinical appearance, and prognosis based on long-term observation. *Blood* **102**(13):4576-4581.

Helenius, A. and M. Aebi. (2001). Intracellular functions of N-linked glycans. *Science* **291**(5512):2364-2369.

Helenius, A. and M. Aebi. (2004). Roles of N-linked glycans in the endoplasmic reticulum. *Ann Rev Biochem* **73**:1019-1049.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **280**(Pt 2):309-316.

Henrissat, B. and A. Bairoch. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem J* **316**(Pt 2):695-696.

Howard, S., Braun, C., McCarter, J., Moremen, K. W., Liao, Y. F. and S. G. Withers. (1997). Human lysosomal and jack bean alpha-mannosidases are retaining glycosidases. *Biochem Biophys Res Commun* **238**(3):896-898.

Ioffe, E. and P. Stanley. (1994). Mice lacking N- acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid N-linked carbohydrates. *Proc Natl Acad Sci U S A* **91**(2):728-732.

Jaeken, J., Carchon, H. and H. Stibler. (1993). The carbohydrate-deficient glycoproteins syndromes: pre-Golgi and Golgi disorder? *Glycobiology* **3**:423-428.

Jaeken, J., Schachter, H., Carchon, H., De Cock, P., Coddeville, B., and G. Spik. (1994). Carbohydrate deficient glycoprotein syndrome type II: a deficiency in Golgi localised N-acetyl-glucosaminyltransferase II. *Arch Dis Child* **71**(2):123-127.

Kaneko, M., Alvarez-Manilla, G., Kamar, M., Lee, I., Lee, J. K., Troupe, K., Zhang, W., Osawa, M. and M. Pierce. (2003). A novel beta(1,6)-N-acetylglucosaminyltransferase V (GnT-VB) (1). *FEBS Lett*. **554**(3):515-559.

Kornfeld, R. and S. Kornfeld (1985). Assembly of asparagine linked oligosaccharides. *Ann Rev Biochem* **54**:631-664.

Koshland, D. E. (1953). Stereochemistry and the mechanism of enzymatic reactions. *Biol Rev Camb Philo Soc* **28**:416-436.

Lal, A., Pang, P., Kalelkar, S., Romero, P. A., Herscovics, A. and K. W. Moremen. (1998). Substrate specificities of recombinant murine Golgi alpha1,2-mannosidases IA and IB and comparison with endoplasmic reticulum and Golgi processing alpha1,2 mannosidases. *Glycobiology* **8**(10):981-995.

Liao, Y. F., Lal, A. and K. W. Moremen. (1996). Cloning, expression, purification, and characterization of the human broad specificity lysosomal acid alpha mannosidase. *J Biol Chem* **271**(45):28348-28358.

Lipari, F. and A. Herscovics. (1994). Production, purification and characterization of recombinant yeast processing alpha 1,2-mannosidase. *Glycobiology* **4**(5): 697-702.

Lipari, F., Gour-Salin, B. J. and A. Herscovics. (1995). The *Saccharomyces cerevisiae* processing alpha 1,2-mannosidase is an inverting glycosidase. *Biochem Biophys Res Commun* **209**(1):322-326.

Lippincott-Schwarz, J., Bonifacino, J. S., Yuan, L. C. and R. D. Klausner. (1988). Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. *Cell* **54**(2):209-220.

Marquadt, T. and J. Denecke. (2003). Congenital disorders of glycosylation: review of their molecular bases, clinical presentations and specific therapies. *Europ J Pediat* **162**:359-379.

Merkle, R. K., Zhang, Y., Ruest, P., J., Lal, A., Liao, Y.-F., and K. W. Moremen. (1997). Cloning, expression, purification, and characterization of the murine lysosomal acid α-mannosidase. *Biochim Biophys Acta* **1336**:132-146.

Metzler, M., Gertz, A., Sarkar, M., Schachter, H., Schrader, J. W. and J. D. Marth. (1994). Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. *EMBO J* **13** (9):2056-65.

Misago, M., Liao, Y. F., Kudo, S., Eto, S., Mattei, M. G., Moremen, K. W. and M. N. Fukuda. (1995). Molecular cloning and expression of cDNAs encoding human alpha-mannosidase II and a previously unrecognized alpha-mannosidase IIx isozyme. *Proc Natl Acad Sci U S A* **92**(25):11766-11770.

Molinari, F., Foulquier, F., Tarpey, P. S., Morelle, W., Boissel, S., Teague, J., Edkins, S., Futreal, P. A., Stratton, M. R., Turner, G., Matthijs, G., Gecz, J., Munnich, A. and L. Colleaux. (2008). Oligosaccharyltransferase-subunit mutations in nonsyndromic mental retardation. *Am J Hum Genet* **82**(5):1150-1157.

Mollicone, R., Moore, S. E., Bovin, N., Garcia-Rosasco, M., Candelier, J. J., Martinez-Duncker, I. and R. Oriol. (2009). Activity, splice variants, conserved peptide motifs, and phylogeny of two new alpha1,3-fucosyltransferase families (FUT10 and FUT11). *J Biol Chem* **284**(7):4723-4738.

Moremen, K. W., Touster, O., and P. W. Robbins. (1991). Novel purification of the catalytic domain of Golgi α-mannosidase II: Characterization and comparison with the intact enzyme. *J. Biol. Chem.* **256**(25):16876-16885.

Moremen, K. W., Trimble, R. B., and A. Herscovics. (1994). Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology* **4**:113-125.

Moremen, K. W. (2000). α-Mannosidases in Asparagine-linked Oligosaccharide Processing and Catabolism. New York:Wiley and Sons.

Moremen, K. W. (2002). Golgi α-mannosidase II deficiency in vertebrate systems: implications for asparagine-linked oligosaccharide processing in mammals. *Biochim Biophys Acta* **1573**(3)**:**225-235.

Nairn, A., York, W. S., Harris, K., Hall, E. M., Pierce, M. J. and K. W. Moremen. (2008). Regulation of glycan structures in animal tissues: TRANSCRIPT PROFILING OF GLYCAN-RELATED GENES. *J Biol Chem* **283**(25):17298-17313.

Ogawa, R., Misago, M., Fukuda, M. N., Kudo, S., Tsukada, J., Morimoto, I. and S. Eto. (1996). Structure and transcriptional regulation of human alpha-mannosidase IIx (alpha-mannosidase II isotype) gene. *Eur J Biochem* **242**(3):446-453.

Oh-Eda, M., Nakagawa, H., Akama, T., Lowitz, K., Misago, M., Moremen, K. W. and M. N. Fukuda. (2001). Overexpression of the Golgi-localized enzyme α-mannosidase IIx in Chinese hamster ovary cells results in the conversion of hexamannosyl-N acetylbhitobiose to tetramannosyl-N-acetylchitobiose in the N-glycan processing pathway. *Eur J Biochem* **268**:1280-1288.

Ohtsubo, K. and J. D. Marth. (2006). Glycosylation in cellular mechanisms of health and disease. *Cell* **126**:855-867.

Oku, H. and S. Hase. (1991) Studies on the substrate specificity of neutral α-mannosidase purified from Japanese quail oviduct by using sugar chains from glycoproteins. *J Biochem* **110**:982-989.

Park, C., Meng, L., Stanton, L. H., Collins, R. E., Mast, S. W., Yi, X., Strachan, H. and K. W. Moremen. (2005) Characterization of a human core-specific lysosomal {alpha}1,6-mannosidase involved in N-glycan catabolism. *J Biol Chem* **280**(44):37204-37216.

Ramaekers, V. T., Stibler, H., Kint, J. and J. Jaeken. (1991). A new variant of the carbohydrate deficient glycoproteins syndrome. *J Inherit Metab Dis* **14**:385-388.

Romero, P. A., Vallee, F., Howell, P. L. and A. Herscovics. (2000). Mutation of Arg(273) to Leu alters the specificity of the yeast N-glycan processing class I alpha 1,2-mannosidase. *J Biol Chem* **275**(15):11071-11074.

Saint-Pol, A., Bauvy, C., Codogno, P. and S. E. H. Moore. (1997). Transfer of free polymannose-type oligosaccharides from the cytosol to lysosomes in cultured human hepatocellular carcinoma HEPG2 cells. *J Cell Biol* **136**(1):45-59.

Shah, N., Kuntz, D. A. and D. R. Rose. (2008). Golgi alpha-mannosidase II cleaves two sugars sequentially in the same catalytic site. *Proc Natl Acad Sci U S A* **105**(28): 9570-9575.

Shoup, V. A. and O. Touster. (1976). Purification and characterization of the alpha-D mannosidase of rat liver cytosol. *J Biol Chem* **251**(13):3845-3852.

Sinnott, M. L. (1990). Catalytic mechanisms of enzymic glycosyl transfer. *Chem Rev* **90**:1171-1202.

Spiro, R. G. (2004). Role of N-linked polymannose oligosaccharides in targeting glycoproteins for endoplasmic reticulum-associated degradation. *Cell Mol Life Sci* **61**(9):1025-1041.

Stanley, P. (1984). Glycosylation mutants of animal cells. *Ann Rev Genet* **18**:525-552.

Strachan, H. (2009). Expression, purification and characterization of a *Drosophila melanogaster* mannosidase homologous to *Drosophila melanogaster* Golgi mannosidase II. Unpublished master's thesis, University of Georgia, Athens, USA.

Suzuki, T., Hara, I., Nakano, M., Shigeta, M., Nakagawa, T., Kondo, A., Funakoshi, Y. and N. Taniguchi. (2006). Man2C1, an α-mannosidase, is involved in the trimming of free oligosaccharides in the cytosol. *Biochem J* **400**:33-41.

Tan, J., D'Agostaro, A. F., Bendiak, B., Reck, F., Sarkar, M., Squire, J. A., Leong, P. and H. Schachter. (1995). The human UDP-N-acetylglucosamine:alpha6-D-mannoside-beta-1,2-N-acetylglucosaminyltransferase II gene (MGAT2). Cloning of genomic DNA, localization to chromosome 14q21, expression in insect cells and purification of the recombinant protein. *Eur J Biochem* **231**(2): 317-328.

Tian, Y., Ju, J. Y., Zhou, Y. Q., Liu, Y., and L. P. Zhu. (2008). Inhibition of alpha-mannosidase Man2c1 expression suppresses growth of esophageal carcinoma cells through mitotic arrest and apoptosis. *Cancer Sci* **99**(12):2428-2434.

Tollersrud, O. K., Berg, T., Healy, P., Evjen, G., Ramachandran, U. and O. Nilssen. (1997). Purification of bovine lysosomal alpha-mannosidase, characterization of its gene and determination of two mutations that cause alpha mannosidosis. *Eur J Biochem* **246**(2):410-419.

Trembley, L. O. and A. Herscovics. (1999). Cloning and expression of a specific human α1,2-mannosidase that trims $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B during N-glycan biosynthesis. *Glycobiology* **9**(10):1073-1078

Tsitilou, S. G. and S. Grammenoudi. (2003). Evidence for alternative splicing and developmental regulation of the *Drosophila melanogaster* Mgat2 (N-acetylglucosaminyltransferase II) gene. *Biochem Biophys Res Commun* **312**(4):1372-1376.

Tulsiani, D. R., Hubbard, S. C., Robbins, P. W. and O. Touster. (1982). Alpha-D-mannosidases of rat liver Golgi membranes. Mannosidase II is the $GlcNAcMan_5$ cleaving enzyme in glycoprotein biosynthesis and mannosidases Ia and IB are the enzymes converting Man_9 precursors to Man_5 intermediates. *J Biol Chem* **257**(7):3660-3668.

Tulsiani, D. R. and O. Touster. (1985). Characterization of a novel alpha-D-mannosidase from rat brain microsomes. *J Biol Chem* **260**(24):13081-13087.

Vallee, F., Karaveg, K., Herscovics, A., Moremen, K. W. and P. L. Howell. (2000). Structural basis for catalysis and inhibition of N-glycan processing class I alpha 1,2-mannosidases. *J Biol Chem* **275**(52):41287-41298.

Vallee, F., Lipari, F., Yip, P., Sleno, B., Herscovics, A. and P. L. Howell. (2000). Crystal structure of a class I alpha1,2-mannosidase involved in N-glycan processing and endoplasmic reticulum quality control. *EMBO J* **19**(4):581-588.

van den Elsen, J. M., Kuntz, D. A. and D. R. Rose. (2001). Structure of Golgi alpha mannosidase II: a target for inhibition of growth and metastasis of cancer cells. *EMBO J* **20**(12):3008-3017.

Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W., and M. E. Etzler, ed. (2009). Essentials of Glycobiology. Woodbury, New York:Cold Spring Harbor Press.

Vembar, S. S. and J. L. Brodsky. (2008). One step at a time: endoplasmic reticulum associated degradation. *Nat Rev Mol Cell Biol* **9**(12):944-957.

Wang, Y., Tan, J., Sutton-Smith, M., Ditto, D., Panico, M., Campbell, R. M., Varki, N. M., Long, J. M., Jaeken, J., Levinson, S. R., Wynshaw-Boris, A., Morris, H. R., Le, D., Dell, A., Schachter, H. and J. D. Marth. (2001). Modeling human congenital disorder of glycosylation type IIa in the mouse: conservation of asparagine-linked glycan dependent functions in mammalian physiology and insights into disease pathogenesis. *Glycobiology* **11**(12):1051-1070.

Weng, S. and R. G. Spiro. (1996). Endoplasmic reticulum kifunensine-resistant α-mannosidase is enzymatically and immunologically related to the cytosolic α-mannosidase. *Arch of Biochem and Biophys* **325**(1):113-123.

Yang, J., Bhaumik, M., Liu, Y. and P. Stanley. (1994). Regulation of N-linked glycosylation. Neuronal cell-specific expression of a 5‘extended transcript from the gene encoding N-acetylglucosaminyltransferase I. *Glycobiology* **4**(5): 703-712.

Zhao, Y.-Y., Takahashi, M., Gu, J.-G., Miyoshi, E., Matsumoto, A., Kitazume, S., and N. Taniguchi. (2008). Functional roles of N-glycans in cell signaling and cell adhesion in cancer. *Cancer Sci.* **99**(7):1304-1310.

Zhong, W., Kuntz, D. A., Ember, B., Singh, H., Moremen, K. W., Rose, D. R., and G. J., Boons. (2008). Probing the substrate specificity of Golgi alpha-mannosidase II by use of synthetic oligosaccharides and a catalytic nucleophile mutant. *J. Am. Chem. Soc.* **130**(28):8975-8983.