

Molecular analysis of the GlcNac-1-phosphotransferase

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Summary Modification of the carbohydrate chains of soluble lysosomal enzymes with mannose 6-phosphate residues is a prerequisite for their mannose 6-phosphate receptor-dependent transport to lysosomes. GlcNac-1-phosphotransferase localized in the Golgi apparatus represents a hexameric $\alpha_2\beta_2\gamma_2$ subunit complex and plays a key role in the formation of the mannose 6-phosphate recognition marker. Defects in the GlcNac-1-phosphotransferase complex cause two diseases, mucopolipidosis type II and III, which are characterized by missorting and cellular loss of lysosomal enzymes, and lysosomal accumulation of storage material. The recent identification of two genes, *GNPTAB* and *GNPTG*, encoding the three subunits of GlcNac-1-phosphotransferase leads to an improvement of both pre- and postnatal diagnosis of affected individuals, and permits the analysis of structural requirements for efficient formation of mannose 6-phosphate residues

on lysosomal enzymes. The α/β subunits precursor matures by proteolytic cleavage and contains the catalytic activity as well as the capability to recognize lysosomal enzymes. The role of the γ -subunits for activity, stability and oligomerization of the GlcNac-1-phosphotransferase subunits is still unclear.

Abbreviations

M6P	mannose 6-phosphate
ML II	mucopolipidosis type II
ML III	mucopolipidosis type III
UDP-GlcNac	UDP- <i>N</i> -acetylglucosamine

Introduction

Newly synthesized lysosomal hydrolases are equipped with mannose 6-phosphate (M6P) residues that function as recognition marker for specific receptors required for lysosomal targeting. The M6P marker is generated in the Golgi apparatus by the sequential action of two enzymes. First, a UDP-*N*-acetylglucosamine (UDP-GlcNac):lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (called GlcNac-1-phosphotransferase) transfers GlcNac-1-phosphate to the C6 hydroxyl group of selected mannoses on high-mannose-type oligosaccharides of lysosomal enzymes. Phosphorylation can be detected at one or two of the five outer mannose residues (Kornfeld and Sly 2001). The number of *N*-linked oligosaccharides that become phosphorylated varies depending on the lysosomal enzyme. In a second step, terminal *N*-acetylglucosamine residues are removed by an *N*-acetylglucosamine-1-phosphodiester α -*N*-acetylglucosaminidase ('uncovering enzyme') exposing the M6P residues (Kornfeld et al 1999).

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M6P-containing enzymes are then recognized by two types of M6P receptors mediating the segregation from the secretory pathway and the transport to a prelysosomal compartment (Storch and Braulke 2005). Defects in the phosphotransferase result in mucopolidosis type II (ML II, I-cell disease) or mucopolidosis type III (ML III, pseudo-Hurler polydystrophy). In patients suffering from these diseases the formation of the M6P recognition marker is impaired, leading to missorting of newly synthesized lysosomal enzymes in the Golgi and subsequent secretion into the extracellular milieu (Kornfeld and Sly 2001).

GlcNac-1-phosphotransferase

The GlcNac-1-phosphotransferase was purified from lactating bovine mammary glands as a 540 kDa heterohexameric complex (Bao et al 1996). The complex is composed of three different subunits α , β , and γ at a 2:2:2 molar ratio. The subunits are encoded by two genes: *GNPTAB* encoding the α/β subunits precursor (Kudo et al 2005; Tiede et al 2005b), and *GNPTG* encoding the γ -subunit (Raas-Rothschild et al 2000).

GNPTAB contains 21 exons and spans 85 kb on chromosome 12q23.3. The encoded protein comprises 1256 amino acids with two putative transmembrane domains (Fig. 1A), and is localized in the *cis* Golgi (Tiede et al 2005b). The α/β subunits precursor contains the catalytic activity of the GlcNac-1-phos-

photransferase (Kudo et al 2005). It has been proposed that the processing of the α/β subunits precursor into mature α - and β -subunits occurs by an unknown protease localized in the endoplasmic reticulum. This protease catalyses a cleavage between Lys⁹²⁹ and Asp⁹³⁰. The proteolytic cleavage is a prerequisite for phosphotransferase activity (Kudo and Canfield 2006). The α/β subunits precursor has a predicted molecular mass of 144 kDa, and the mature α - and β -subunits have masses of 105 and 39 kDa, respectively. The proposed α - and β -subunits contain 16 and 3 potential *N*-glycosylation sites, respectively. The cDNA sequence of the α/β phosphotransferase gene is conserved in human, mouse, rat, chicken and zebrafish. Sequence comparisons showed that the luminal domain of the α/β -subunits precursor has a complex modular structure comprising at least six domains (Tiede et al 2005b). An N-terminal luminal domain similar to bacterial capsule biosynthesis proteins with an additional inserted domain might contain the UDP-GlcNac binding site or could be involved in the transfer of GlcNac-1-phosphate (Fig. 1B). There are two domains in the α -subunit that are related to Notch receptor repeats, and another domain exhibits similarity to the domain that binds the transcriptional co-repressor DMAP1 (Tiede et al 2005b). The sequence comprising the mature luminal β -subunit is highly conserved between human, mouse and chicken. The impact of these domains on catalytic activity, oligomerization of subunits, and interaction with other proteins remains to be investigated.

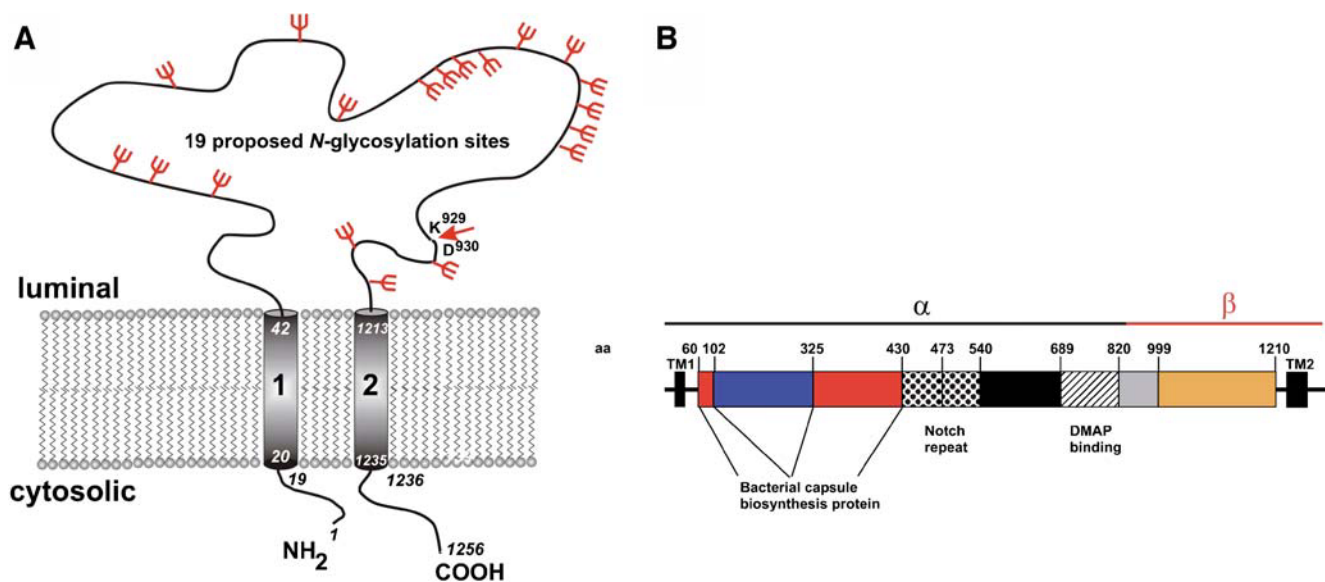


Fig. 1 (A) Proposed topology of the α/β subunits precursor of the GlcNac-1-phosphotransferase spanning the membrane twice. The luminal domain contains 19 potential *N*-glycosylation sites. The proposed proteolytic cleavage site between Lys⁹²⁹ and Asp⁹³⁰ is indicated. (B) Domain organization of the α/β subunits precursor. The numbering of amino acids (aa) is given. TM, transmembrane domain.

GNPTG

GNPTG contains 11 exons and spans 11.13 kb on chromosome 16p13.3. It encodes a protein of 305 amino acids. After cleavage of the 24-amino-acid signal peptide, the mature protein forms disulfide-linked homodimers and becomes glycosylated at Asn⁸⁸ and Asn¹¹⁵ (Raas-Rothschild et al 2000; Tiede et al 2004). It has been believed that the γ -subunit of GlcNac-1-phosphotransferase functions in recognition of lysosomal enzymes (Raas-Rothschild et al 2000). Analysis of M6P-containing lysosomal enzymes in γ -subunit-deficient mouse cells, however, suggests that both α/β - as well as γ -subunits interact with lysosomal enzymes but with different protein determinants (Lee et al 2007). Alternatively, γ -subunits might facilitate the proper folding of α/β -subunits or maintain them in a conformation competent for enzyme recognition and binding. These data are in agreement with our own studies using surface plasmon resonance spectrometry demonstrating that recombinant γ -subunits do not bind the recombinant lysosomal arylsulfatase A. Furthermore, overexpression of human γ -subunits in HEK293 or COS7 cells did not increase the secretion of four newly synthesized lysosomal enzymes tested. This would be expected if sequestration of the enzymes by an excess of γ -subunits prevented M6P residue attachment by the endogenous GlcNac-1-phosphotransferase (Tiede et al, manuscript in preparation). Finally, the failure of lysosomal proteins to bind to a γ -subunit affinity matrix (Tiede et al 2005b) supported the view that γ -subunits are not involved in interactions with specific substrates.

Molecular basis of mucopolidoses

GlcNac-1-phosphotransferase activity is deficient in two distinct autosomal recessive human diseases, ML II and ML III. In fibroblasts of patients with ML II demonstrating the I-cell phenotype with prominent cytoplasmic inclusions, phosphotransferase activity is absent, leading to the loss of intracellular lysosomal enzyme activities (Kornfeld and Sly 2001). ML II patients are characterized by dwarfism, skeletal abnormalities, developmental delay and cardiomegaly leading to death between 5 and 8 years of age (Spranger et al 2002). ML III is an attenuated form of the disorder with later onset of clinical symptoms and more slowly progressive course allowing survival into the eighth decade (Umehara et al 1997). Defective phosphotransferase leads to a wide spectrum of disorders ranging from lethal prenatal forms (Pacman dysplasia) (Saul et al

2005), mucopolipidosis with severe neonatal hyperparathyroidism (Unger et al 2005), to attenuated patients diagnosed as having mucopolipidosis III (Freisinger et al 1992). No causal therapies are available for the treatment of ML patients. GlcNac-1-phosphotransferase activity is decreased in cells of patients with ML III. Genetic complementation studies led to subdivision of ML III into three distinct complementation groups, ML IIIA–C (Little et al 1986). Group B, however, was represented by only a single cell line with unknown molecular defect. Initially three ML IIIC families have been described carrying a single base insertion in the *GNPTG* gene resulting in frameshift and early termination (Raas-Rothschild et al 2000). Seven other *GNPTG* mutations including missense, frameshift and splice mutations have been reported recently (Raas-Rothschild et al 2004; Tiede et al 2004). Almost all patients with classical severe ML II are associated with nonsense or frameshift mutations in the *GNPTAB* gene (Bargal et al 2006; Kudo et al 2006; Paik et al 2005; Plante et al 2008; Tappino et al 2008; Tiede et al 2005b). However, an increasing number of reports of clinically diagnosed ML III patients being homozygous or compound heterozygous for mutations in the *GNPTAB* gene (Bargal et al 2006; Paik et al 2005; Steet et al 2005; Tiede et al 2005a, 2006) confirms predictions that ML II and ML IIIA are caused by the same gene defect (Honey et al 1982). To prevent confusion in classification of mucopolipidosis patients based on clinical symptoms or gene defects, a reclassification has been proposed summarizing our understanding of clinical, biochemical and molecular heterogeneity of mucopolipidosis II and III. Thus, ML II is proposed to become ML II alpha/beta; ML IIIA to become ML III alpha/beta, and ML IIIC to become ML III gamma (Cathey et al 2008).

Animal models of ML II

Two animal models of ML II are known. A colony of domestic short-hair cats is described that displays several clinical and radiographic features similar to the human disease. However, diffuse retinal degeneration leading to blindness by 4 months of age develops in affected kittens, which does not appear to be a typical feature in ML II patients. The breeding of affected cats is difficult owing to the variability in lifespan, ranging from 1 day to 216 days (Mazrier et al 2003). Recently, the murine *GNPTAB* gene was disrupted by insertion of a gene-trapping retroviral vector in intron 1 (Gelfman et al 2007). The affected mice are smaller and exhibit cartilage defects, and exhibit severe retinal degeneration

leading to loss of photoreceptor function by 5 months of age. The animal models will provide valuable tools for investigation of the pathogenesis of ML II and the development of novel therapeutic strategies for this disease.

Summary and conclusion

Recent work led to the identification of the *GNPTAB* gene encoding the α/β subunits precursor that contains the catalytic activity of the GlcNac-1-phosphotransferase. This enzyme plays a key role in the formation of mannose 6-phosphate residues on lysosomal enzymes required for efficient transport to lysosomes. Defects in the GlcNac-1-phosphotransferase cause two lysosomal storage diseases, mucopolipidosis types II and III. Knowledge of the disease-causing genes will improve pre- and postnatal diagnosis of affected individuals. Mutational analyses of ML II and complementation subtype ML IIIA patients revealed that both diseases are caused by the same gene defect resulting in a wide spectrum of clinical phenotypes, and a reclassification of mucopolipidosis subtypes has been proposed. The functions of distinct domains of the GlcNac-1-phosphotransferase α/β subunit precursor in catalytic activity, oligomerization of subunits, and interaction with other proteins remain to be investigated. The availability of animal models of mucopolipidosis II will provide new insights into the pathomechanisms of the disease.

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