

ORIGINAL ARTICLE

Analyses of disease-related GNPTAB mutations define a novel GlcNAc-1-phosphotransferase interaction domain and an alternative site-1 protease cleavage site

Renata Voltolini Velho^{1,†}, Raffaella De Pace^{2,†}, Sarah Klünder²,
Fernanda Sperb-Ludwig³, Charles Marques Lourenço⁴,
Ida V. D. Schwartz^{1,3}, Thomas Braulke² and Sandra Pohl^{2,*}

¹Department of Genetics, Universidade Federal do Rio Grande do Sul, Avenida Paulo Gama 110, 90040-060 Porto Alegre, Brazil, ²Department of Biochemistry, Children's Hospital, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany, ³Laboratory of Basic Research and Advanced Investigations in Neurosciences, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcellos 2350, 90035-903 Porto Alegre, Brazil and ⁴Neurogenetics Unit, Department of Neurology, School of Medicine of Ribeirao Preto, University of São Paulo, Avenida Bandeirantes 3900, 14050-260 Ribeirão Preto, Brazil

*To whom correspondence should be addressed. Tel: +49 40741058780; Fax: +49 40741058504; Email: s.pohl@uke.de

Abstract

Mucopolidosis II (MLII) and III alpha/beta are autosomal-recessive diseases of childhood caused by mutations in GNPTAB encoding the α/β -subunit precursor protein of the GlcNAc-1-phosphotransferase complex. This enzyme modifies lysosomal hydrolases with mannose 6-phosphate targeting signals. Upon arrival in the Golgi apparatus, the newly synthesized α/β -subunit precursor is catalytically activated by site-1 protease (S1P). Here we performed comprehensive expression studies of GNPTAB mutations, including two novel mutations T644M and T1223del, identified in Brazilian MLII/MLIII alpha/beta patients. We show that the frameshift E757KfsX1 and the non-sense R587X mutations result in the retention of enzymatically inactive truncated precursor proteins in the endoplasmic reticulum (ER) due to loss of cytosolic ER exit motifs consistent with a severe clinical phenotype in homozygosity. The luminal missense mutations, C505Y, G575R and T644M, partially impaired ER exit and proteolytic activation in accordance with less severe MLIII alpha/beta disease symptoms. Analogous to the previously characterized S399F mutant, we found that the missense mutation I403T led to retention in the ER and loss of catalytic activity. Substitution of further conserved residues in stealth domain 2 (I346 and W357) revealed similar biochemical properties and allowed us to define a putative binding site for accessory proteins required for ER exit of α/β -subunit precursors. Interestingly, the analysis of the Y937_M972del mutant revealed partial Golgi localization and formation of abnormal inactive β -subunits generated by S1P which correlate with a clinical MLII phenotype. Expression analyses of mutations identified in patients underline genotype–phenotype correlations in MLII/MLIII alpha/beta and provide novel insights into structural requirements of proper GlcNAc-1-phosphotransferase activity.

[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Received: February 10, 2015. Revised and Accepted: March 13, 2015

© The Author 2015. Published by Oxford University Press.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Introduction

Lysosomal enzymes catalyze the degradation of a variety of endogenous and exogenous macromolecules. They require mannose 6-phosphate (M6P) residues on their oligosaccharides for efficient targeting of lysosomes. The formation of M6P is initiated in the lumen of the cis-Golgi apparatus by the transfer of an N-acetylglucosamine (GlcNAc)-1-phosphate from UDP-GlcNAc to distinct mannose residues generating GlcNAc-1-phosphodiester (1–3). This reaction is catalyzed by a hexameric GlcNAc-1-phosphotransferase complex consisting of two α -, β - and γ -subunits (4). In a second step, the masking GlcNAc residues are removed in the trans-Golgi network by the uncovering enzyme that exposes terminal M6P modifications (5). The M6P residues allow the binding of newly synthesized lysosomal enzymes to M6P-specific receptors which mediate their segregation from the secretory pathway and delivery to the endosomal/lysosomal compartment (6).

The α - and β -subunits of GlcNAc-1-phosphotransferase complex are synthesized as a common α/β -subunit precursor protein encoded by the *GNPTAB* gene that is located on chromosome 12q23.3 (7,8). The *GNPTG* gene, located on chromosome 16p13.3, encodes the γ -subunit (9). Non-sense, frameshift and splice site mutations in *GNPTAB* cause the severe lysosomal storage disorder mucopolidiosis type II (MLII; MIM #252500; also called I-cell disease) which is associated with a complete loss of GlcNAc-1-phosphotransferase activity (10). In contrast, missense mutations in *GNPTAB* showing residual GlcNAc-1-phosphotransferase activity result in a milder course of the disease (MLIII alpha/beta; MIM #252600) (10,11). The MLIII gamma form (MIM #252605) is caused by defects in the *GNPTG* gene (11). To date, more than 140 different *GNPTAB* mutations have been described.

Biochemically, the complete or partial loss of the GlcNAc-1-phosphotransferase activity leads to missorting and hypersecretion of multiple lysosomal enzymes. The subsequent intracellular deficiency of lysosomal enzymes results in the accumulation of non-degraded storage material in lysosomes (12). MLII patients suffer from severe psychomotor retardation and show coarse facial features, gingival hypertrophy, shortened neck, joint contractures, osteopenia, *dysostosis multiplex* and very short stature. Death occurs due to cardiopulmonary complications within the first decade of life (11,13). MLIII alpha/beta is presented by progressive joint stiffness, claw hands, carpal and tarsal tunnel syndrome, scoliosis and decreased mobility of knees and hip joints (11).

The α/β -subunit precursor is a type III membrane protein of 1256 amino acids and two transmembrane domains. Its exit from the endoplasmic reticulum (ER) requires a combinatorial sorting motif located in the N- and C-terminal cytoplasmic tails (14). Upon arrival in the cis-Golgi apparatus, the α/β -subunit precursor is proteolytically cleaved between amino acids K928 and D929 into the mature α - and β -subunits by the site-1 protease (S1P), which also plays an essential role in cholesterol homeostasis (15,16). The proteolytic cleavage of α/β -subunit precursor protein is a prerequisite for the catalytic activity of the GlcNAc-1-phosphotransferase and therefore plays an important role in the biogenesis of lysosomes.

The luminal α -subunit exhibits a conserved modular structure, and recently the DNA methyltransferase-associated protein (DMAP) domain has been described as the binding site for lysosomal enzymes (17). The α - and β -subunits mediate the catalytic function of GlcNAc-1-phosphotransferase (16), which appears to be modulated by the γ -subunits (18).

Here, we used the subcellular localization determined by double immunofluorescence microscopy, and the capability to be enzymatically activated by the Golgi-localized S1P to analyze the

effect of eight selected disease-causing *GNPTAB* mutations found in Brazilian MLII and MLIII alpha/beta patients. We have presented evidence that in addition to the loss of combinatorial cytosolic targeting motifs (14), luminal missense mutations located in the 'stealth' region 2 of the α -subunit impair the transport of the α/β -subunit precursor to the Golgi apparatus. This region most likely represents a contact site for a yet unknown accessory transport protein. Finally, a stretch of amino acids in the N-terminus of the β -subunit is essential for precise S1P-mediated cleavage and activity of the GlcNAc-1-phosphotransferase.

Results

We examined the biological significance of eight selected disease-causing *GNPTAB* mutations found in MLII and MLIII alpha/beta patients in Brazil. Two of the mutations, T644M and T1223del, were novel and found in heterozygosity in the patient described in Materials and Methods (Supplementary Material, Table S1, patient #6). In addition, three missense mutations (I403T, C505Y and G575R), an in-frame deletion mutation of 36 amino acids (Y937_M972del), a non-sense mutation (R587X) and a frameshift mutation (E757KfsX1), which were identified previously (19,20) have been included in this study (Fig. 1). These mutations were introduced into the wild-type (WT) cDNA of human *GNPTAB*-myc constructs and transiently expressed in HEK-293, HeLa or S1P-deficient SRD-12B cells. The cells were analyzed for expression, subcellular localization of the GlcNAc-1-phosphotransferase and correlated to the activity.

Expression of mutant α/β -subunits of the GlcNAc-1-phosphotransferase

First of all, cell extracts were analyzed by western blot using a monoclonal antibody raised against the α -subunit (21) or

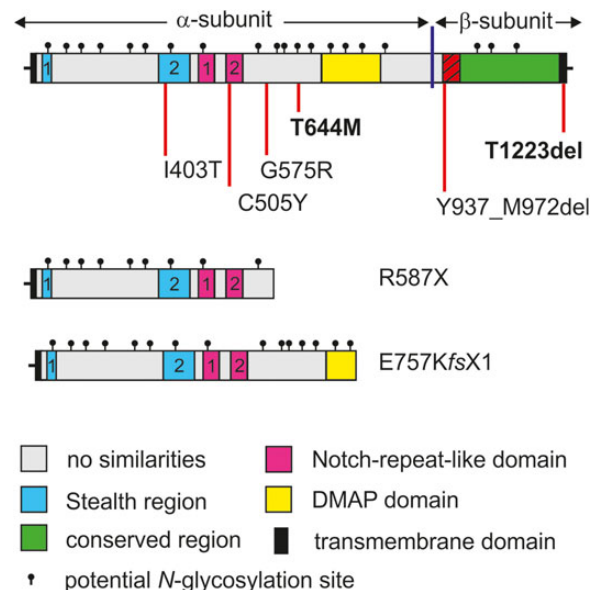


Figure 1. Schematic presentation of the domain organization of the full-length WT and mutant α/β -subunit precursor protein of the GlcNAc-1-phosphotransferase. The positions of the four missense and two deletion mutations are indicated in the upper scheme. The novel mutations identified in the MLIII alpha/beta patient are marked in bold. The C-terminally truncated α/β -subunit precursor mutants R587X and E757KfsX1 are shown below. The positions of potential N-glycosylation sites (filled circle) and the S1P cleavage site between amino acid K928 and D929 (in blue) are indicated.

myc-tag to detect the α - and β -subunits, respectively, as well as the α/β -subunit precursor proteins of human GlcNAc-1-phosphotransferase. In cells expressing the WT protein, a 190 kDa α/β -subunit precursor and 45 kDa β -subunits were detectable (Fig. 2A, lane 2, B, lane 1) which were not present in extracts of non-transfected control cells (Fig. 2A, lane 1). The reactivity of the monoclonal antibody with the mature α -subunit of a calculated molecular mass of 145 kDa was weak, most likely due to the high N-glycosylation, which could be improved by deglycosylation of the samples. After treatment of cell extracts with peptide-N-glycosidase F (PNGase F), hydrolyzing all N-linked oligosaccharides, the molecular masses of the α/β -subunit precursor and α - and β -subunits shifted to ~170, 125 and 38 kDa (Fig. 2A, lane 3, B, lane 2), respectively. In extracts of cells overexpressing the missense mutant I403T, the α/β -subunit precursor protein but no cleaved α - and β -subunits were observed (Fig. 2A, lanes 4 and 5). The mutants C505Y, G575R (Fig. 2A, lanes 6–9) and T644M (Fig. 2B, lanes 3 and 4) were cleaved into mature α - and β -subunits. Densitometric analysis revealed, however, that only 20, 10 and 30% of the C505Y, G575R and T644M mutants were proteolytically processed in comparison to 80% of the WT α/β -subunit precursor. The non-sense R587X mutant led to the expression of a truncated 70 kDa glycosylated and 60 kDa non-glycosylated polypeptide which could be detected with the anti α -subunit antibody but not with the anti-myc antibody (Fig. 2A, lanes 10 and 11). The frameshift mutant E757KfsX1 was expressed as 120 kDa glycosylated and 80 kDa non-glycosylated polypeptide (Fig. 2B, lanes 5 and 6). In extracts from HEK-293 cells overexpressing the Y937_M972del mutant, the loss of 36 amino acid residues was hardly detectable by changes in the electrophoretic mobility of the α/β -subunit precursor (Fig. 2B, lanes 7 and 8). However, we unexpectedly observed no β -subunits with increased electrophoretic mobility due to the deletion but

with approximately the same mass as the WT β -subunit (Fig. 2B, lanes 7 and 8). In contrast, we observed in other experiments even a lower migrating β -subunit (discussed below). The mutant T1223del α/β -subunit precursor was cleaved into mature α - and β -subunits (Fig. 2B, lanes 9 and 10).

Densitometric analysis of western blots from five independent experiments revealed that the expression levels of the deletion mutants were comparable to the WT polypeptide, whereas the amounts of all other mutants were reduced by 50–75% (Fig. 3A). To determine whether variations of mutant α/β -subunit precursor protein result from differences in transcript levels, the mRNA levels of overexpressed WT and mutant GNPTAB constructs were analyzed by real-time polymerase chain reaction (PCR). The transcript levels of the four missense and two deletion mutants were comparable to WT (Fig. 3B), whereas the amounts of R587X and E757KfsX1 mRNA were decreased by 40%, suggesting that these mutant mRNAs are unstable (Fig. 3B).

Subcellular localization of mutant GlcNAc-1-phosphotransferase

Proteolytic S1P-mediated activation of the α/β -subunit precursor of GlcNAc-1-phosphotransferase into mature subunits occurs in the cis-Golgi apparatus (8,21). To examine the subcellular localization of mutant GlcNAc-1-phosphotransferase subunits, HeLa cells overexpressing WT or mutant α/β -subunit precursor polypeptides were analyzed by immunofluorescence microscopy using anti α -subunit antibodies. Cells were co-stained for the ER marker protein disulfide isomerase (PDI) or the cis-Golgi marker protein GM130. WT α/β -subunits were found to co-localize with GM130 but not with PDI (Fig. 4). In accordance with the lack of processing of the mutant α/β -subunit precursor I403T (Fig. 2A), it completely co-localized with PDI (Fig. 4). The majority of the

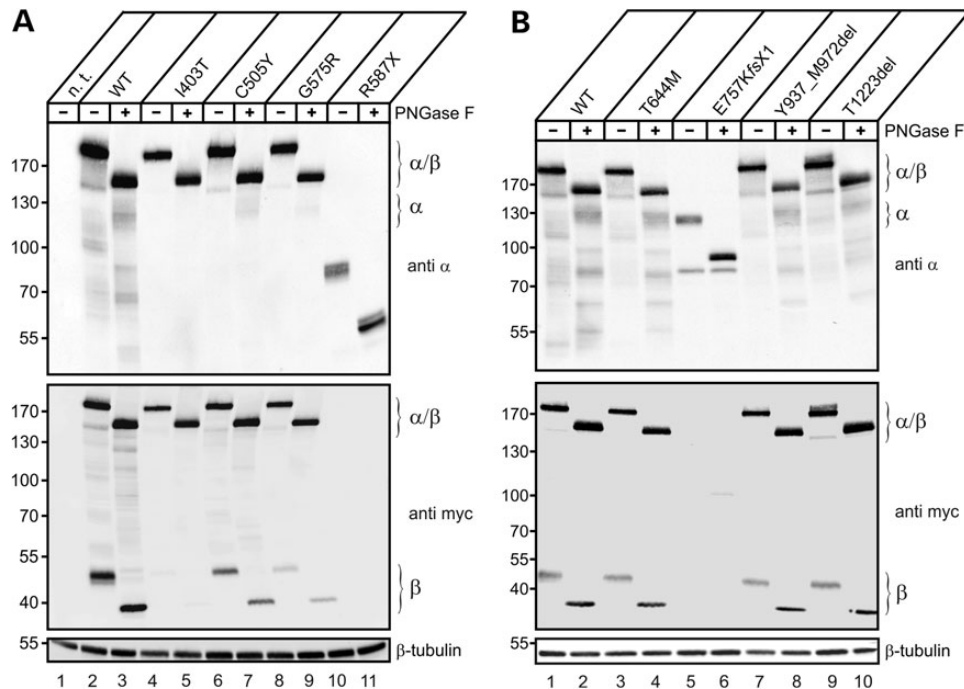


Figure 2. Expression and proteolytic cleavage of WT and mutant α/β -subunit precursor constructs of GlcNAc-1-phosphotransferase. (A, B) HEK-293 cells were transfected with cDNA encoding WT or the indicated mutant α/β -subunit precursor constructs. Twenty-four hours after transfection, cell extracts were incubated for 1 h in the presence (+) or absence (-) of PNGase F followed by SDS-PAGE (10% acrylamide) under reducing conditions and western blot analysis detecting α/β -subunit precursor and α -subunits (anti α) or α/β -subunit precursors and β -subunits (anti-myc). β -Tubulin and extracts of non-transfected cells were used as loading and negative control, respectively. The positions of molecular mass marker proteins (in kDa), α/β -subunit precursor, α - and β -subunits are indicated.

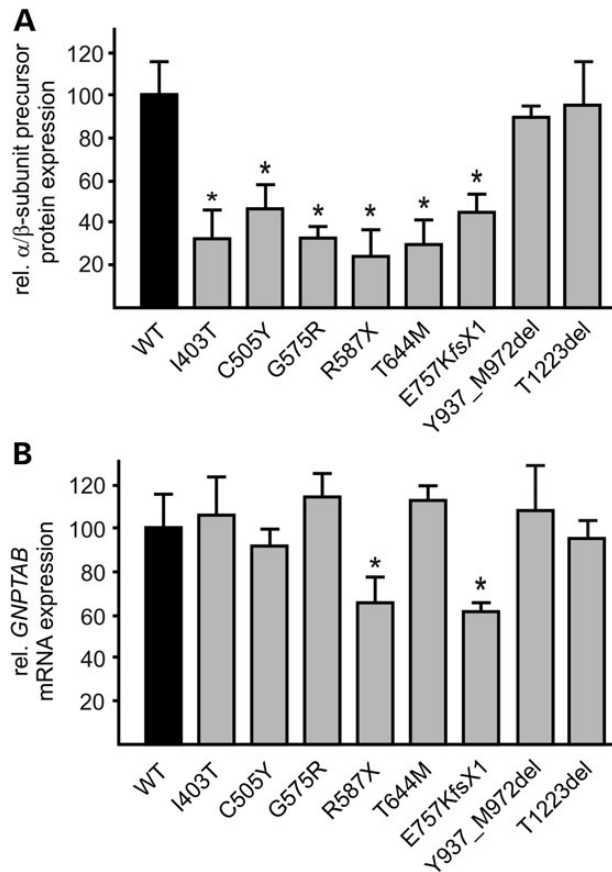


Figure 3. Relative expression of mutant α/β -subunit precursor GlcNAc-1-phosphotransferase. (A) Densitometric evaluation of western blots analyzing the mutated α/β -subunit precursor protein expression from five independent transfections were performed and normalized to β -tubulin level. The expression of the WT α/β -subunit precursor was set as 100%. (B) The relative mRNA levels of overexpressed WT and mutant GNPTAB were determined by real-time PCR and normalized to ACTB mRNA expression (mean \pm SEM, $n = 5$, * $P < 0.05$). The mRNA expression of WT α/β -subunit precursor corrected by the endogenous GNPTAB in HEK-293 cells was assigned as 100%.

C505Y, G575R and R587X mutants co-localize with PDI and only a small amount of the G575R mutant reached GM130-positive Golgi structures (Fig. 4). As expected by the presence of cleaved α - and β -subunits (Fig. 2B), the mutant T644M was correctly targeted to the Golgi apparatus and showed co-localization with GM130 but not with PDI (Fig. 4). The frameshift mutant E757KfsX1 and the deletion mutant Y937_M972del completely co-localized with PDI. Of note, when the mutant Y937_M972del has been expressed in HEK-293 cells, partial co-staining with the Golgi marker protein GM130 was observed (Supplementary Material, Fig. S2). The deletion mutant T1223del was correctly transported to the cis-Golgi apparatus and showed co-localization with GM130 (Fig. 4).

Enzymatic activity of mutant GlcNAc-1-phosphotransferase

The type of mutation in the GNPTAB gene and the position of the mutation in the α/β -subunit precursor protein affect the GlcNAc-1-phosphotransferase activity and determine the clinical phenotype of the patients (11,22). To examine genotype-phenotype correlations in this cohort of Brazilian MLII and MLIII alpha/beta patients, the GlcNAc-1-phosphotransferase activity was measured using α -methylmannoside (α -MM) as a

phosphate acceptor. The expression of the WT α/β -subunits precursor of GlcNAc-1-phosphotransferase has led to a 13-fold increase in GlcNAc-1-phosphotransferase activity compared with non-transfected cells. Less than 2% of WT GlcNAc-1-phosphotransferase activity was measured in cells expressing R587X and E757KfsX1 mutants (Fig. 5) which are retained in the ER (Fig. 4). The activities of mutant GlcNAc-1-phosphotransferase I403 T, C505Y and G575R were also significantly reduced to 4–6% of WT. The deletion of 36 amino acids in the β -subunit (Y937_M972del) led to a complete ER localization in HeLa cells (Fig. 4), and a partial localization in the Golgi apparatus in HEK-293 cells (Supplementary Material, Fig. S2) associated with an irregularly cleaved α/β -subunit precursor protein showed total loss of GlcNAc-1-phosphotransferase activity (Fig. 5). The mutants T644M and T1223del that were correctly transported and proteolytically cleaved into mature α - and β -subunits exhibited 50 and 85% of GlcNAc-1-phosphotransferase activity, respectively, of the WT enzyme. When the GlcNAc-1-phosphotransferase activities of mutated forms were related to the amounts of β -subunits determined by densitometric evaluation of western blots (Supplementary Material, Fig. S3), a similar activity pattern was observed, indicating that C505Y, G575R, T644M and T1223del show residual activity of 7, 3, 50 and 85%, respectively, whereas Y937_M972del (as well as all mutants retained in the ER) led to the total loss of GlcNAc-1-phosphotransferase activity. These data are consistent with the milder and less progressive MLIII alpha/beta than MLII phenotype of the patients carrying the T644M and T1223del mutations.

Defining a novel luminal ER export domain in the α -subunit

In addition to mutant I403T, another close-by mutation, S399F, has been described that is also located in the highly conserved stealth domain 2 of the α -subunit (Supplementary Material, Fig. S4) and prevent the exit from the ER and the subsequent S1P-mediated cleavage in the Golgi apparatus (21). To examine whether other residues in this domain play a similar role for ER exit, six conserved amino acid residues located proximal or distal to S399/I403 were selected randomly (I346, W357, L380, E389, D408, and Y421) and substituted by alanine (Supplementary Material, Fig. S4). The expression analysis revealed that all mutants are stable (Fig. 6). However, the mutant α/β -subunit precursor proteins W357A and I346A were not or only partially cleaved, respectively, by S1P (Fig. 6, lanes 3 and 4). All other tested mutants were cleaved by S1P and could exit the ER. The data suggest that at least amino acid residues at positions I346, W357, S399 and I403 in the stealth domain 2 play an important role for the export of the α/β -subunit precursor from the ER. Most likely, these residues form an interaction site for protein(s) promoting the α/β -subunit precursor trafficking to the Golgi apparatus.

S1P-mediated abnormal cleavage of the Y937_M972del mutant GlcNAc-1-phosphotransferase

To analyze whether the irregular cleavage of the Y937_M972del mutant α/β -subunit precursor was catalyzed by S1P, the mutant construct was expressed in S1P-deficient SRD-12B CHO cells. The loss of S1P resulted in a reduction of the endogenous GlcNAc-1-phosphotransferase activity by 75% of the parental control CHO cells (Fig. 7A). Residual activity of GlcNAc-1-phosphotransferase is caused by the non-homogeneous composition of the SRD-12B cells due to incomplete amphotericin B selection (23). Re-expression of S1P in SRD-12B cells rescued the cleavage of

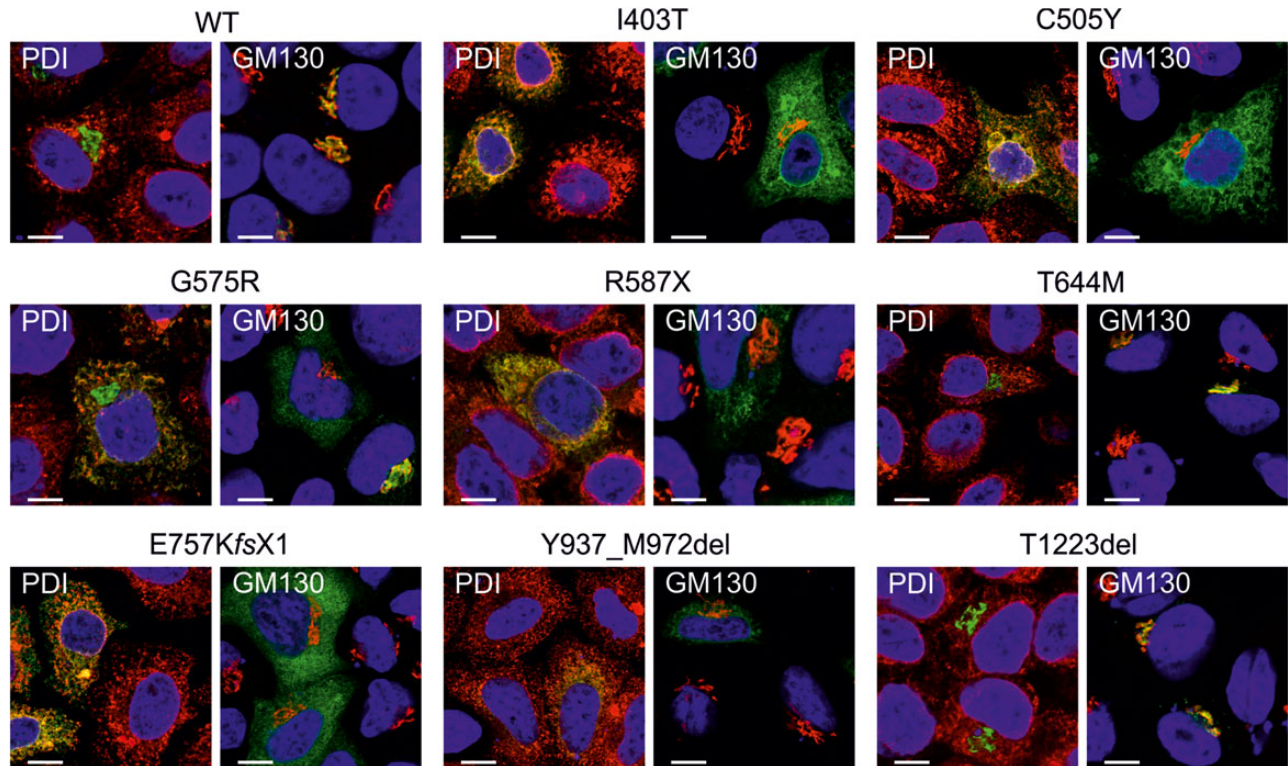


Figure 4. Intracellular localization of WT and mutant α/β -subunit precursor constructs of GlcNAc-1-phosphotransferase. HeLa cells were transfected with cDNA encoding WT or mutant α/β -subunit precursor constructs. Prior fixation, cells were treated with 100 $\mu\text{g}/\text{ml}$ cycloheximide for 40 min to block further protein translation and allow protein export from the ER, followed by staining with monoclonal antibodies against the α -subunit (green), the cis-Golgi marker protein GM130 (red) or the ER marker protein PDI (red). Nuclei were visualized by DAPI staining (blue). Magnified views of merged images (indicated by rectangles in Supplementary Material; Fig. S1) are shown. Yellow indicates colocalization. Scale bar: 5 μm .

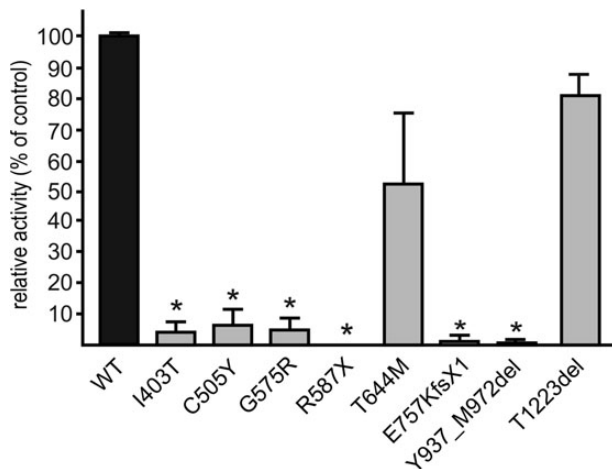


Figure 5. GlcNAc-1-phosphotransferase activity of WT and mutant α/β -subunit precursor constructs. The activity towards α -MM corresponding to 100 μg protein extracts of HEK-293 cells overexpressing full-length WT or mutant α/β -subunit constructs was measured for 60 min. The activity of overexpressed WT (9034 ± 1566 pmol/mg/h corrected by the endogenous GlcNAc-1-phosphotransferase activity) was set to 100%. Data are average values of three independent experiments, and error bars represent SEM. * $P < 0.005$.

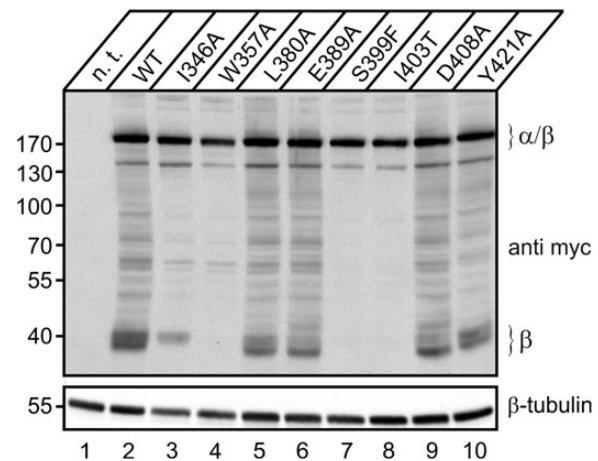


Figure 6. Proteolytic cleavage of luminal α/β -subunit precursor mutants of GlcNAc-1-phosphotransferase. HEK-293 cells were transfected with cDNA encoding WT or the indicated mutant α/β -subunit precursor constructs. Twenty-four hours after transfection, cell extracts were analyzed by SDS-PAGE (10% acrylamide) under reducing conditions and western blot detecting α/β -subunit precursor and β -subunit (anti-myc). β -Tubulin and extracts of non-transfected cells were used as loading and negative control, respectively. The positions of molecular mass marker proteins (in kDa), α/β -subunit precursor and β -subunits are indicated.

the α/β -subunit precursor (15) and the activity of endogenous GlcNAc-1-phosphotransferase activity to 75% of control CHO cells (Fig. 7A and B). Western blot analysis revealed that the non-glycosylated Y937_M972del mutant α/β -subunit precursor

exhibits a slightly faster mobility than the WT protein in extracts of both SRD-12B cells and HEK-293 cells used as positive control (Fig. 8). Surprisingly, a reduction rather than an increased

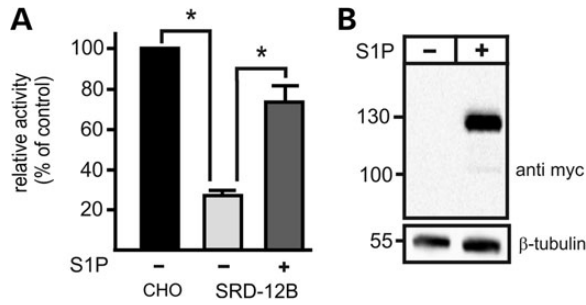


Figure 7. Endogenous GlcNAc-1-phosphotransferase activity in S1P-deficient cells. (A) The activity towards α -MM corresponding to 100 μ g protein extracts of SRD-12B and CHO cells was measured for 60 min. The activity in CHO cells was set to 100%. Overexpression of myc-tagged human S1P rescued GlcNAc-1-phosphotransferase activity. Data are average values of three independent experiments, and error bars represent SEM. * $P < 0.05$. (B) SRD-12B cells were transfected with cDNA encoding human myc-tagged S1P. Twenty-four hours after transfection, cell extracts were analyzed by SDS-PAGE (10% acrylamide) under reducing conditions and western blot against the myc-tag of S1P. β -Tubulin western blot was used as a loading control. Extracts of non-transfected SRD-12B cells were used as negative control. The positions of molecular mass marker proteins (in kDa) are indicated.

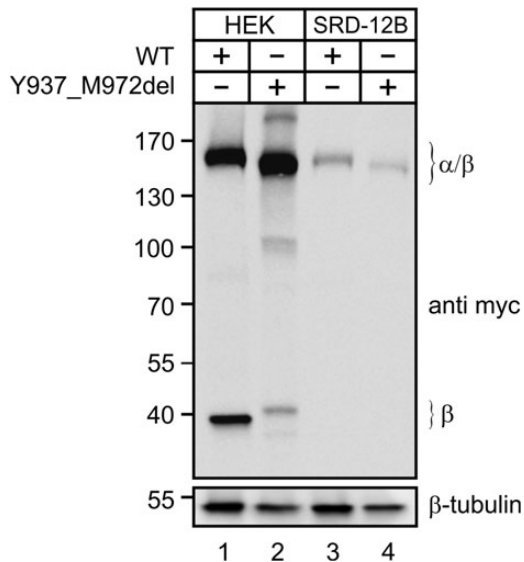


Figure 8. Proteolytic cleavage of Y937_M972del mutant α/β -subunit precursor. HEK-293 and SRD-12B cells were transfected with cDNA encoding WT or mutant α/β -subunit precursor constructs. Twenty-four hours after transfection, cell extracts were prepared and incubated for 1 h in the presence of PNGase F, followed by SDS-PAGE (8–16% acrylamide gradient) under reducing conditions and western blot analysis of the β -subunit (anti-myc). β -Tubulin western blot was used as a loading control. The positions of molecular mass marker proteins (in kDa) are indicated.

electrophoretic mobility of the β -subunits was observed, suggesting the generation of a new cleavage site located in the α -subunit. In contrast to HEK-293 cells (Fig. 8, lanes 1 and 2), no cleavage of the Y937_M972del mutant could be observed in SRD-12B cells (Fig. 8, lanes 3 and 4). The data demonstrate that the abnormal cleavage of the Y937_M972del mutant α/β -subunit precursor is mediated by S1P, but fails to activate the GlcNAc-1-phosphotransferase.

Discussion

The comprehensive expression analyses of mutations identified in the GNPTAB gene of Brazilian patients allow predictions on the

severity and clinical course of the diseases, MLII and MLIII alpha/beta, and provide novel insights into the complex domain structure of α/β -subunits of GlcNAc-1-phosphotransferase and their role in substrate binding and catalytic activity. Several molecular defects result in the total loss of GlcNAc-1-phosphotransferase activity and severe MLII, such as mutations affecting (i) the catalytic center, (ii) UDP-GlcNAc or lysosomal enzyme binding sites, (iii) the transport of the α/β -subunit precursors from the ER to the Golgi apparatus and (iv) the capability of the α/β -subunit precursors to be proteolytically cleaved by S1P in the cis-Golgi cisternae. At present, 33 of the 144 known GNPTAB mutations have been studied by expression analysis in HeLa or HEK-293 cells (14,17,21,24,25).

Here we show that the mutations leading to a frameshift and the synthesis of C-terminally truncated α/β -subunit precursor forms, R587X, E757KfsX1 and L1168QfsX5, cause the complete loss of GlcNAc-1-phosphotransferase activity and a severe MLII phenotype in homozygosity (patient #1, Supplementary Material, Table S1) or in compound heterozygosity with another deleterious mutation (patient #2). The loss of ER exit signals in the cytoplasmic domain of the β -subunit (14) in these truncated α/β -subunit precursors is responsible for the retention in the ER and the subsequent inability for S1P-mediated proteolytic activation. In contrast, both mutations T644M and T1223del identified in the patient #6 altered the amino acid sequence in the region between the Notch-repeat-like domain 2 and DMAP domain and the second transmembrane domain (Fig. 1), respectively, partially impaired Golgi localization, proteolytic activation and GlcNAc-1-phosphotransferase activity. These data are consistent with the milder course of the disease and justify the classification as MLIII alpha/beta (Supplementary Material, Table S1). Of note, the substitution of T644 by methionine changes a potential N-glycosylation in the α -subunit. At present, it is unclear whether N642 is glycosylated *in vivo* and the loss of this N-glycosylation site affects the function of the GlcNAc-1-phosphotransferase complex.

The 31-year-old MLIII alpha/beta patient #5 was found to be compound heterozygous for the non-sense and missense mutations R587X and C505Y, respectively. The premature termination after R586 in the α -subunit explains both the ER localization and the absence of GlcNAc-1-phosphotransferase activity. The substitution, however, of cysteine 505 by tyrosine in the Notch-repeat-like domain 2 (Fig. 1) impaired the ER-Golgi transport and proteolytic activation which is consistent with the residual GlcNAc-1-phosphotransferase activity (Supplementary Material, Table S1). During the preparation of the manuscript, Kornfeld and co-workers (25) reported the analysis of two missense mutations affecting cysteine residues in the Notch-repeat-like domain 2 of the α -subunit. Our data are in agreement with their finding on the C505Y mutation. Although comparative activity measurements in cysteine mutants of the Notch-repeat-like domain 1 (C442Y, C461G and C468S) suggested that this domain is involved in the recognition of lysosomal enzyme binding (25), it is unclear whether the Notch-repeat-like domain 2 harboring C505 residue also has a role in lysosomal enzyme binding.

We previously reported that the mutation S399F located in the stealth domain 2 (Fig. 1) is retained in the ER and not cleaved to mature α - and β -subunits (21). Interestingly, the Brazilian patient #3 carries the heterozygous mutation I403T that behaves in an analogous manner as S399F (21) (Figs 2 and 4) lacking catalytic activity as shown by Kornfeld and co-workers (25). When we examined the substitutions of other randomly selected residues by alanine, I346A and W357A were found to be not or partially cleaved by S1P (Fig. 6). Two other mutations in the stealth domain

2 have been reported, R334Q and R334L (10,26), which are localized in the ER and therefore lack cleavage and catalytic activity (25). Thus, it is likely that at least the amino acid residues R334, I346, W357, S399 and I403 in stealth domain 2 form a putative surface structure capable of interacting with a yet unknown protein in the lumen of the ER required for proper sorting to the ER exit sites, cargo incorporation into the nascent vesicles and/or transport to the Golgi apparatus. Several proteins functioning as subunits or luminal chaperones in the trafficking of transporter proteins or transcription factors from the ER to other organelles have been described (27–30). The identification of potential accessory proteins which bind to signal structures of the stealth domain 2 in the α -subunit is currently under investigation.

Of particular interest was the Y937_M972del mutant which was partially transported to the Golgi apparatus and abnormally cleaved (Fig. 8). The regular cleavage site of the α/β -subunit precursor between K928 and D929 is located in the consensus recognition motif of S1P (R/K)X(hydrophobic)Z₁, where X represents any amino acid and Z preferentially L or T. For cleavage, at least 20 amino acid residues proximal to the cleavage site are required to allow the interaction of S1P with the α/β -subunit precursor protein (15). Obviously, an intact amino acid sequence covering more than nine residues distal to the cleavage site is also necessary for effective S1P-mediated cleavage. Although no further S1P consensus sequences in the α -subunit are known, the experiments in S1P-deficient SRD-12B cells have demonstrated that S1P is the responsible protease for the generation of abnormal cleavage of the α -subunit preventing the proper activation step. The identification of the abnormal cleavage site remains to be investigated.

The data presented here demonstrate the importance of comprehensive expression analyses of missense and deletion mutations in the GNPTAB gene in the context with a thorough clinical evaluation and help to define genotype–phenotype correlations in order to improve the predictions of the clinical course of MLII and III alpha/beta. In addition, the expression analysis represents an important approach to gain new insights into the composition and complexity of the modular domain structure of the α - and β -subunits, as well as in the function of the GlcNAc-1-phosphotransferase and the homeostasis of lysosomes.

Materials and Methods

Reagents

Penicillin/streptomycin (P/S), α -methylmannoside (α -MM), adenosine 5'-triphosphate (ATP) disodium salt, uridine 5'-diphosphate N-acetylglucosamine (UDP-GlcNAc) sodium salt, bovine serum albumin (BSA), 4',6'-diamidino-2-phenylindol (DAPI), protease inhibitor cocktail, cholesterol, sodium mevalonate, sodium oleate, amphotericin B and other common laboratory reagents were obtained from Sigma. UDP-[³H]GlcNAc was purchased from American Radiolabeled Chemicals. QAE™ Sephadex A-25 was from GE Healthcare. Fetal bovine serum (FBS) was from PAA Laboratories. Easy-DNA gDNA Purification Kit, Dulbecco's modified Eagle's medium (DMEM), F12 Ham and GlutaMAX™ were from Life Technologies. Transfection reagent JetPEI® was purchased from Peqlab. Phusion® polymerase and GeneJET Plasmid Miniprep Kit, GeneJET PCR Purification Kit, dNTPs and prestained protein ladder Page-ruler™ were from Thermo Scientific. Oligonucleotides used for sequencing and mutagenesis were synthesized by MWG Biotech. PNGase F was from Roche Applied Sciences. Mowiol® and Roti®quant Protein Assay were from Roth. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gradient gels (8–16%) from NuSep were used.

Antibodies

The monoclonal rat antibody against the human α -subunit of the GlcNAc-1-phosphotransferase was described recently (21). Monoclonal antibodies against myc-tag, PDI and GM130 were purchased from Cell Signalling, Biomol and BD Biosciences, respectively. The monoclonal antibody against β -tubulin was obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies conjugated to HRP, anti-mouse Alexa Fluor® 546 and anti-rat Alexa Fluor® 488 were purchased from Dianova and Life Technologies, respectively.

Clinical manifestation, biochemical and genetic diagnosis of an MLIII alpha/beta patient

The male patient (#6, Supplementary Material, Table S1) was the only son of non-consanguineous parents of Brazilian origin. The full-term pregnancy was carried with no problems occurring. Birth weight (3610 g), length (50 cm) and head circumference were normal. He first presented at 1 year and 6 months of age with stiffness of small hand and finger joints. At the age of 2 years, he had a generalized bone dysplasia with thoracic deformities, coarse facies, depressed nasal bridge, scoliosis, *pectus carinatum* and claw hands, as well as gingival hypertrophy and language delay. There is no history of recurrent infections. Neither myopenia nor hearing problems were found. The activities of α -L-iduronidase, iduronate 2-sulfatase and β -glucuronidase in leukocytes were normal excluding MPSI, II and VII. Strongly elevated activities in plasma and reduced activities of several lysosomal enzymes in cultured fibroblasts of the patient led to the diagnosis of MLII or MLIII. Genomic sequencing of GNPTAB and GNPTG revealed no alterations in GNPTG, whereas the patient was found to be heterozygous for the novel mutations c.1931_1932CA>TG (T644M) and c.3668_3670delCTA (T1223del) and therefore diagnosed as MLIII alpha/beta. The patient was re-evaluated at 9 years. At that time, he attended regular school and had no cognitive impairment. At physical examination, he presented with 97 cm of height (<3 percentile) and 17 kg of weight (<3 percentile), thickening of metopic suture, claw hands joint contractures of shoulders, elbows, hands hip and knees, and radiological evidence of *dysostosis multiplex*. There were no corneal clouding or organomegaly, but the boy had diathesis of the *rectus abdominis*, leading to abdominal protuberance. Ophthalmological examination was normal. There was mitral insufficiency on echocardiogram.

GNPTAB/GNPTG mutational analysis

Genomic DNA from the patient (#6, Supplementary Material, Table S1) and his parents were extracted from peripheral blood leukocytes with the Easy-DNA DNA Purification Kit, and the 21 or 11 exons that comprise the GNPTAB or GNPTG gene, respectively, were amplified as described previously (19,31). Sample sequencing was performed using the automatic ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequences of the GNPTG and GNPTAB genes used as reference was GenBank accession numbers NG_016985.1 and NM_024312.4, respectively.

Generation of mutant GNPTAB cDNA constructs

The pathogenic GNPTAB mutations c.1208T>C (I403T), c.1514G>A (C505Y), c.1723G>A (G575R), c.1759C>T (R587X), c.1931_1932CA>TG (T644M), c.2269_2273delGAAAC (E757KfsX1), c.3668_3670delCTA (T1223del) (Fig. 1) as well as structural GNPTAB mutations (I346A, W357A, L380A, E389A, D408A and Y421A) were inserted into the

C-terminally myc-tagged full-length WT GNPTAB construct (14) by site-directed mutagenesis using mutagenic primers and Phusion[®] polymerase. Mutagenic primers were designed using the web-based program PrimerX (www.bioinformatics.org/primerx). The mutation c.2808A>G was not associated with an amino acid change but effect the splicing. This mutation creates a similar sequence to the canonical donor splice site, and consequently cDNA analysis of the patient RNA revealed the presence of an abnormal transcript lacking the last 108 bp of exon 14 resulting in an in-frame deletion of 36 amino acids of the β -subunit (Y937_M972del) (19). To introduce this mutation into the GNPTAB construct, 48 bp primers were used. The generation of mutant GNPTAB construct S399F was described recently (21). All nucleotide primers used for cloning and site-directed mutagenesis are listed in Supplementary Material, Table S2. The DNA plasmids were commercially sequenced to confirm proper introduction of the mutations (SeqLab). Mutation nomenclature follows the HSVG rules and is based on GenBank accession number NM_024312.4, with nucleotides numbered using 1 as the A of the ATG starting codon.

Cell culture and transfections

HEK-293 and HeLa cells were maintained in DMEM supplemented with 10% FBS, GlutaMAX[™] and P/S at 37°C and 5% CO₂. SRD-12B cells and their parental CHO control cells were cultured in a 1:1 mixture of DMEM and nutrient mixture F12 Ham with 5% FCS and P/S. SRD-12B cells were supplemented with 5 μ g/ml cholesterol, 50 μ M sodium mevalonate and 20 μ M sodium oleate. Weekly selection with amphotericin B of SRD-12B cells was performed as described elsewhere (23).

Cells grown on 6-cm plates or on glass cover slips were transiently transfected with cDNAs coding for human WT or mutant α / β -subunit precursor-myc fusion proteins using JetPEI[®] reagent according to the manufacturer's instructions. All constructs were transfected in parallel under identical conditions, and cell extracts were analyzed by western blot, immunofluorescence microscopy, mRNA expression and GlcNAc-1-phosphotransferase activity 24 h after transfection.

GlcNAc-1-phosphotransferase activity assays

HEK-293 cells as well as SRD-12B and their parental CHO control cells were lysed in PBS containing 1% Triton X-100 and protease inhibitor cocktail for 15 min on ice. After centrifugation at 10 000g, supernatants were used for measurement of the protein content by the Roti[®]quant Protein Assay. Aliquots of cell extracts (100 μ g protein) were adjusted to 50 mM Tris-HCl (pH 7.4) containing 10 mM MgCl₂, 10 mM MnCl₂, 2 mg/ml BSA, 2 mM ATP, 75 μ M UDP-GlcNAc and incubated with 1 μ Ci UDP-[³H]GlcNAc and 100 mM α -MM at 37°C for 1 h (24). After incubation, 1 ml of 2 mM Tris-HCl (pH 8.0) was added to the samples and applied to QAE sephadex A-25 column (1 ml of resin equilibrated with 2 mM Tris-HCl, pH 8.0) and the columns were washed twice with 2 ml and once with 1 ml of 2 mM Tris-HCl (pH 8.0). The bound [³H] GlcNAc-P reaction product was eluted twice with 2 ml and once with 1 ml of 30 mM NaCl in 2 mM Tris-HCl (pH 8.0). Each elution was collected and measured in 5 vol. of scintillation liquid. The values obtained from non-transfected cell lysates were subtracted from the values obtained from cell lysates transfected with WT or the mutants to correct for endogenous enzyme activity and non-specific background counts. Depending on the mutation, the GlcNAc-1-phosphotransferase activity was related to the steady-state expression of the β -subunit determined by densitometric

analysis of western blots using Imager ChemiDoc XRS (Biorad) and Image Lab software.

Other methods

Isolation of total RNA, cDNA synthesis, and real-time PCR was performed as described previously (32). Confocal microscopy of transfected cells, preparation of cell extracts, enzymatic deglycosylation of proteins, and SDS-PAGE followed by western blot analysis were performed as described recently (21).

Statistical analysis

Results are expressed as mean \pm SEM. Differences between mean values are determined using Student's t-test. P-values <0.05 were considered significant.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

The authors would like to thank Johannes Brand, Taciane Alegra, Maira Burin and Nataniel Ludwig for expert technical assistance, and the UKE Microscopy Imaging Facility for support.

Conflict of Interest statement. None declared.

Funding

This work was supported by Deutscher Akademischer Austauschdienst and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq to R.V.V.), by Deutsche Forschungsgemeinschaft (GRK1459 to R.D.P., SFB877/B3 to S.K., S.P. and T.B.) and by Fundação de Amparo à Pesquisa do Rio Grande do Sul (post-doctorate fellowship to F.S.L.). Funding to pay the Open Access publication charges for this article was provided by Deutsche Forschungsgemeinschaft.

References

1. Reitman, M.L. and Kornfeld, S. (1981) UDP-N-acetylglucosamine: glycoprotein N-acetylglucosamine-1-phosphotransferase. Proposed enzyme for the phosphorylation of the high mannose oligosaccharide units of lysosomal enzymes. *J. Biol. Chem.*, **256**, 4275–4281.
2. Waheed, A., Pohlmann, R., Hasilik, A. and von Figura, K. (1981) Subcellular location of two enzymes involved in the synthesis of phosphorylated recognition markers in lysosomal enzymes. *J. Biol. Chem.*, **256**, 4150–4152.
3. Pohl, S., Marschner, K., Storch, S. and Bräulke, T. (2009) Glycosylation- and phosphorylation-dependent intracellular transport of lysosomal hydrolases. *Biol. Chem.*, **390**, 521–527.
4. Bao, M., Booth, J.L., Elmendorf, B.J. and Canfield, W.M. (1996) Bovine UDP-N-acetylglucosamine:lysosomal-enzyme N-acetylglucosamine-1-phosphotransferase. I. Purification and subunit structure. *J. Biol. Chem.*, **271**, 31437–31445.
5. Kornfeld, R., Bao, M., Brewer, K., Noll, C. and Canfield, W. (1999) Molecular cloning and functional expression of two splice forms of human N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase. *J. Biol. Chem.*, **274**, 32778–32785.
6. Bräulke, T. and Bonifacino, J.S. (2009) Sorting of lysosomal proteins. *Biochim. Biophys. Acta*, **1793**, 605–614.

7. Kudo, M., Bao, M., D'Souza, A., Ying, F., Pan, H., Roe, B.A. and Canfield, W.M. (2005) The alpha- and beta-subunits of the human UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase [corrected] are encoded by a single cDNA. *J. Biol. Chem.*, **280**, 36141–36149.
8. Tiede, S., Storch, S., Lübke, T., Henrissat, B., Bargal, R., Raas-Rothschild, A. and Braulke, T. (2005) Mucopolipidosis II is caused by mutations in GNPTA encoding the alpha/beta GlcNAc-1-phosphotransferase. *Nat. Med.*, **11**, 1109–1112.
9. Raas-Rothschild, A., Cormier-Daire, V., Bao, M., Genin, E., Salomon, R., Brewer, K., Zeigler, M., Mandel, H., Toth, S., Roe, B. et al. (2000) Molecular basis of variant pseudo-hurler polydystrophy (mucopolipidosis IIIC). *J. Clin. Invest.*, **105**, 673–681.
10. Cathey, S.S., Leroy, J.G., Wood, T., Eaves, K., Simensen, R.J., Kudo, M., Stevenson, R.E. and Friez, M.J. (2010) Phenotype and genotype in mucopolipidoses II and III alpha/beta: a study of 61 probands. *J. Med. Genet.*, **47**, 38–48.
11. Braulke, T., Raas-Rothschild, A. and Kornfeld, S. (2013) I-cell disease and pseudo-Hurler polydystrophy: disorder of lysosomal enzyme phosphorylation and localization. In Valle, D., Vogelstein, B., Kinzler, K.W., Antonarakis, S.E., Ballabio, A., Scriver, C.R., Sly, W.S., Bunz, F., Gibson, K.M. and Mitchell, G. (eds), *The Online Metabolic and Molecular Basis of Inherited Diseases*. McGraw-Hill, NY, chapter 138. www.ommbid.com (last accessed, February 2013).
12. Kollmann, K., Pohl, S., Marschner, K., Encarnaçao, M., Sakwa, I., Tiede, S., Poorthuis, B.J., Lübke, T., Müller-Loennies, S., Storch, S. et al. (2010) Mannose phosphorylation in health and disease. *Eur. J. Cell Biol.*, **89**, 117–123.
13. Spranger, J., Brill, P. and Poznanski, A. (2002) Oxford University Press, New York, pp. 57–79.
14. Franke, M., Braulke, T. and Storch, S. (2013) Transport of the GlcNAc-1-phosphotransferase alpha/beta-subunit precursor protein to the Golgi apparatus requires a combinatorial sorting motif. *J. Biol. Chem.*, **288**, 1238–1249.
15. Marschner, K., Kollmann, K., Schweizer, M., Braulke, T. and Pohl, S. (2011) A key enzyme in the biogenesis of lysosomes is a protease that regulates cholesterol metabolism. *Science*, **333**, 87–90.
16. Kudo, M. and Canfield, W.M. (2006) Structural requirements for efficient processing and activation of recombinant human UDP-N-acetylglucosamine:lysosomal-enzyme-N-acetylglucosamine-1-phosphotransferase. *J. Biol. Chem.*, **281**, 11761–11768.
17. Qian, Y., Flanagan-Steet, H., van Meel, E., Steet, R. and Kornfeld, S.A. (2013) The DMAP interaction domain of UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase is a substrate recognition module. *Proc. Natl Acad. Sci. USA*, **110**, 10246–10251.
18. Qian, Y., Lee, I., Lee, W.S., Qian, M., Kudo, M., Canfield, W.M., Lobel, P. and Kornfeld, S. (2010) Functions of the alpha, beta, and gamma subunits of UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase. *J. Biol. Chem.*, **285**, 3360–3370.
19. Cury, G.K., Matte, U., Artigas, O., Alegria, T., Velho, R.V., Sperb, F., Burin, M.G., Ribeiro, E.M., Lourenco, C.M., Kim, C. A. et al. (2013) Mucopolipidosis II and III alpha/beta in Brazil: analysis of the GNPTAB gene. *Gene*, **524**, 59–64.
20. Sperb-Ludwig, F., Alegria, T., Voltolini, R., Ludwig, N., Kim, C.A., Kok, F., Kitajima, J.P., van Meel, E., Kornfeld, S.A., Burin, M.G. et al. (2015) Exome sequencing for mucopolipidosis III: GNPTAB gene novel mutation in a patient with a milder phenotype. *Mol. Genet. Metab. Rep.*, **2**, 34–37.
21. De Pace, R., Coutinho, M.F., Koch-Nolte, F., Haag, F., Prata, M.J., Alves, S., Braulke, T. and Pohl, S. (2014) Mucopolipidosis II-related mutations inhibit the exit from the endoplasmic reticulum and proteolytic cleavage of GlcNAc-1-phosphotransferase precursor protein GNPTAB. *Hum. Mutat.*, **35**, 368–376.
22. Kudo, M., Brem, M.S. and Canfield, W.M. (2006) Mucopolipidosis II (I-cell disease) and mucopolipidosis IIIA (classical pseudo-hurler polydystrophy) are caused by mutations in the GlcNAc-phosphotransferase alpha/beta-subunits precursor gene. *Am. J. Hum. Genet.*, **78**, 451–463.
23. Rawson, R.B., Cheng, D., Brown, M.S. and Goldstein, J.L. (1998) Isolation of cholesterol-requiring mutant Chinese hamster ovary cells with defects in cleavage of sterol regulatory element-binding proteins at site 1. *J. Biol. Chem.*, **273**, 28261–28269.
24. van Meel, E., Qian, Y. and Kornfeld, S.A. (2014) Mislocalization of phosphotransferase as a cause of mucopolipidosis III alpha/beta. *Proc. Natl Acad. Sci. USA*, **111**, 3532–3537.
25. Qian, Y., van Meel, E., Flanagan-Steet, H., Yox, A., Steet, R. and Kornfeld, S. (2015) Analysis of mucopolipidosis II/III GNPTAB missense mutations identifies domains of UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase involved in catalytic function and lysosomal enzyme recognition. *J. Biol. Chem.*, **290**, 3045–3056.
26. Otomo, T., Muramatsu, T., Yorifuji, T., Okuyama, T., Nakabayashi, H., Fukao, T., Ohura, T., Yoshino, M., Tanaka, A., Okamoto, N. et al. (2009) Mucopolipidosis II and III alpha/beta: mutation analysis of 40 Japanese patients showed genotype-phenotype correlation. *J. Hum. Genet.*, **54**, 145–151.
27. Lange, P.F., Wartosch, L., Jentsch, T.J. and Fuhrmann, J.C. (2006) ClC-7 requires Ostm1 as a beta-subunit to support bone resorption and lysosomal function. *Nature*, **440**, 220–223.
28. Paulusma, C.C., Folmer, D.E., Ho-Mok, K.S., de Waart, D.R., Hilarius, P.M., Verhoeven, A.J. and Oude Elferink, R.P. (2008) ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. *Hepatology*, **47**, 268–278.
29. Das, S., Smith, T.D., Sarma, J.D., Ritzenthaler, J.D., Maza, J., Kaplan, B.E., Cunningham, L.A., Suaud, L., Hubbard, M.J., Rubenstein, R.C. et al. (2009) ERp29 restricts Connexin43 oligomerization in the endoplasmic reticulum. *Mol. Biol. Cell.*, **20**, 2593–2604.
30. Shen, J., Snapp, E.L., Lippincott-Schwartz, J. and Prywes, R. (2005) Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response. *Mol. Cell Biol.*, **25**, 921–932.
31. Persichetti, E., Chuzhanova, N.A., Dardis, A., Tappino, B., Pohl, S., Thomas, N.S., Rosano, C., Balducci, C., Paciotti, S., Dominissini, S. et al. (2009) Identification and molecular characterization of six novel mutations in the UDP-N-acetylglucosamine-1-phosphotransferase gamma subunit (GNPTG) gene in patients with mucopolipidosis III gamma. *Hum. Mutat.*, **30**, 978–984.
32. Pohl, S., Tiede, S., Castrichini, M., Cantz, M., Gieselmann, V. and Braulke, T. (2009) Compensatory expression of human N-acetylglucosaminyl-1-phosphotransferase subunits in mucopolipidosis type III gamma. *Biochim. Biophys. Acta*, **1792**, 221–225.