Subunit interactions of the disease-related hexameric GlcNAc-1-phosphotransferase complex

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Abstract

The multimeric GlcNAc-1-phosphotransferase complex catalyzes the formation of mannose 6-phosphate recognition marker on lysosomal enzymes required for receptor-mediated targeting to lysosomes. GNPTAB and GNPTG encode the α/β-subunit precursor membrane proteins and the soluble γ-subunits, respectively. Performing extensive mutational analysis, we identified the binding regions of γ-subunits in a previously uncharacterized domain of α-subunits comprising residues 535–698, named GNPTG binding (GB) domain. Both the deletion of GB preventing γ-subunit binding and targeted deletion of GNPTG led to significant reduction in GlcNAc-1-phosphotransferase activity. We also identified cysteine 70 in α-subunits to be involved in covalent homodimerization of α-subunits which is, however, required neither for interaction with γ-subunits nor for catalytic activity of the enzyme complex. Finally, binding assays using various γ-subunit mutants revealed that residues 130–238 interact with glycosylated α-subunits suggesting a role for the mannose 6-phosphate receptor homology domain in α-subunit binding. These studies provide new insight into the assembly of the GlcNAc-1-phosphotransferase complex, and the functions of distinct domains of the α- and γ-subunits.

Introduction

Mucolipidosis II (MLII, MIM#252500, also called I-cell disease) and MLIII (MIM#252600) are autosomal recessive disorders of lysosomal enzyme targeting caused by defects in the N-acetylglucosamine (GlcNAc)-1-phosphotransferase. This enzyme catalyzes the first step in the formation of mannose 6-phosphate (M6P) recognition marker on lysosomal enzymes required for their efficient M6P receptor-mediated transport to lysosomes (1). GlcNAc-1-phosphotransferase comprises a 540 kDa hexameric complex consisting of two α-subunits, two β-subunits and two γ-subunits (2). The α- and β-subunits of this enzyme complex are encoded by the GNPTAB gene [MIM #607840; (3,4)] whereas the γ-subunit is encoded by GNPTG [MIM #607838; (5)]. Mutations in GNPTAB resulting in complete or partial loss of GlcNAc-1-phosphotransferase activity lead to severe (MLII alpha/beta) or attenuated (MLIII alpha/beta) clinical course of the disease in children. Patients with mutations in GNPTG (MLIII gamma) have a milder progression permitting survival into adulthood (6,7).

The α- and β-subunits are synthesized in the endoplasmic reticulum (ER) as a common 190-kDa type III precursor membrane protein associated with folding, N-glycosylation, disulfide-linked homodimerization and assembly with soluble 36-kDa γ-subunits (2,8). Upon signal-dependent transport of the enzymatic inactive GlcNAc-1-phosphotransferase complex to the Golgi apparatus (9), site-1 protease catalyzes the cleavage of the α/β-subunit precursor protein between residues K928 and D929 into mature 145-kDa type II α- and 45-kDa type I β-subunit.
membrane proteins [Fig. 1A; (10)]. The cleaved α- and β-subunits mediate the catalytic function of the GlcNAc-1-phosphotransferase, although the role of the individual subunits remains to be defined (11). Studies of γ-subunit-deficient mice suggested that γ-subunits modulate the phosphorylation efficiency of subsets of lysosomal enzymes (12). Little is known on the role of various preserved modular structures composing the luminal part of the α-subunit membrane protein. This part consists of two stealth domains similar to bacterial capsule biosynthesis proteins (13), two Notch-repeat-like domains of unknown function and a DNA methyltransferase-associated protein (DMAP) domain, which has been reported to bind lysosomal proteins (14). These domains are separated by domains showing no similarities with other proteins (Fig. 1A).

In the present study, we have analyzed the structural requirements for the assembly of the GlcNAc-1-phosphotransferase complex. Pull-down experiments of wild-type (WT) and various mutant α/β-subunit precursor fusion proteins with γ-subunits were performed and allowed the identification of interacting subunits and the defined binding region. The data demonstrate that the γ-subunits bind to a glycosylated region in the no-similarity domain 2 of the α-subunit, which is independent on cysteine 70 identified to be responsible for α-subunit homodimerization. The loss of γ-subunit binding led to a reduction of GlcNAc-1-phosphotransferase activity.

Results

Interaction of γ-subunits with glycosylated α-subunits

Previously, we have shown that the α/β-subunit precursor protein of GlcNAc-1-phosphotransferase binds to the immobilized γ-subunit (4). To identify the binding site of γ-subunit in the α- or β-subunit, γ-subunit-GFP pull-down assays have been performed. C-terminally tagged γ-subunit-GFP fusion constructs were generated, expressed in human embryonic kidney (HEK) 293 cells and precipitated using GFP-Trap® beads. As control, GFP alone has been precipitated from overexpressing cells. The preformed GFP-Trap® complexes were incubated with detergent extracts of HEK-293 cells overexpressing GFP-α/β-myc (C) or γ-GFP (D) and E) were incubated with GFP-Trap® beads. After washing, the preformed complexes were incubated with extracts of HEK-293 cells overexpressing α/β-myc (C and D) or HA-β (E). Non-bound material was separated by centrifugation in the supernatant (S). Aliquots of the input (IN, 10%), supernatants (S, 10%), the last wash fractions (W, 10%) and the bound material collected by solubilization of the GFP-Trap® bead complexes (B, 100%) were resolved by SDS-PAGE followed by GFP and myc western blotting. The positions of the molecular mass marker proteins in kilodaltons and the γ-subunit-GFP and GFP are indicated.

Figure 1. Interaction between α/β-subunit precursor and γ-subunit of GlcNAc-1-phosphotransferase. [A] Schematic representation of the α/β-subunit precursor membrane protein, cleaved by site-1 protease (red arrow) between K928 and D929 into mature α- and β-subunits, and the soluble γ-subunit. The luminal domain of the α-subunit contains stealth domain 1 and 2 [turquoise, amino acids (aa) 75–86 and 322–421, respectively], Notch-repeat-like domains 1 and 2 (magenta, aa 359–453 and 500–553, respectively), DMAP domain (yellow, aa 699–814) and no-similarity domains 1, 2 and 3 (gray, aa 873–212, 536–698 and 815–928, respectively). The numbering starts at the first methionine of the coding sequence. The potential N-glycosylation sites (filled circles) are indicated. (B) Schematic representation of α/β-myc, γ-GFP, GFP alone and HA-β used in these experiments. The apparent molecular masses of the constructs in kilodaltons are given. (C–E) Extracts of HEK-293 cells overexpressing GFP (C) or γ-GFP (D and E) were incubated with GFP-Trap® beads. After washing, the preformed complexes were incubated with extracts of HEK-293 cells overexpressing α/β-myc (C and D) or HA-β (E). Non-bound material was separated by centrifugation in the supernatant (S). Aliquots of the input (IN, 10%), supernatants (S, 10%), the last wash fractions (W, 10%) and the bound material collected by solubilization of the GFP-Trap® bead complexes (B, 100%) were resolved by SDS-PAGE followed by GFP and myc western blotting. The positions of the molecular mass marker proteins in kilodaltons and the α/β-subunit precursor (α/β-myc), β-subunits (β-myc or HA-β), γ-subunits (γ-GFP) and GFP are indicated.
β-my subunit (Fig. 1C). Neither the α/β-my nor the β-my subunits bound to GFP-bead complexes (Fig. 1C). When γ-GFP pull-down experiments were performed with α/β-my-containing extracts, both the glycosylated 190-kDa α/β-my precursor and the 45-kDa β-my subunits were found in the γ-GFP-bound fraction (Fig. 1D). Similarly, 220-kDa full-length α/β-k-GFP fusion proteins were able to precipitate the human 36-kDa non-tagged γ-subunits from extracts of overexpressing cells (Supplementary Material, Fig. S1). To examine whether the β-subunit interacts with γ-GFP, single HA-tagged β-subunits were expressed which failed, however, to bind to γ-GFP (Fig. 1E). These data suggest that γ-subunit interacts directly with the α-subunit of the GlcNAc-1-phosphotransferase. Of note, the β-my subunit observed in the γ-GFP-bound fraction is most likely due to co- incidental binding to the precipitated non-tagged α-subunit.

Next C-terminally tagged single α-my subunits were expressed in HEK-293 cells as 130- and 100-kDa polypeptides (Fig. 2A and B, input). PNGase F digestion of α-my-expressing cell extracts leads to the complete loss of N-linked oligosaccharides and the conversion of the 130-glycosylated into the 100-kDa non-glycosylated α-my subunit (Supplementary Material, Fig. S2). Only the 150-kDa glycosylated α-my form was found to bind γ-GFP (Fig. 2B). As it is known that mutations in GNPTG changing the N-linked glycosylation of the γ-subunit cause MLIII gamma (15), we examined whether non-glycosylated γ-subunits still interact with α-subunits. Pull-down experiments revealed that γ-subunits in which both N-glycosylation sites at N88 and N115 have been substituted by glutamine bind to the same extent as the WT γ-subunits to α/β-GFP and α-subunits (Fig. 2C), indicating that N-linked oligosaccharides on γ-subunits are not needed for their interaction with α-subunits.

### γ-subunits bind to no-similarity domain 2

To define the region in the α-subunit that binds γ-subunits, three deletion mutants were generated (Fig. 3A). In the first construct, the amino acids 431–848 were deleted comprising the Notch-repeat-like domains 1 and 2, the no-similarity domain 2 and the DMAP domain (α/β-my Δ431–848). The second and third constructs lack both Notch-repeat-like domains 1 and 2 (α/β-my Δ433–535), and the DMAP domain (α/β-my Δ698–814), respectively (Fig. 3A). Transfection of HEK-293 cells with mutant α/β-my cDNAs resulted in the expression of 120-, 170- and 165-kDa α/β-my subunit precursor proteins, respectively. With the exception of α/β-my Δ433–535, α/β-my Δ431–848 and Δ698–814 are cleaved into mature β-my subunits indicating their proper folding, export from the ER and S1P-mediated cleavage in the Golgi apparatus (Fig. 3B). Subsequently, γ-GFP-Trap® complexes were incubated with cell extracts containing the individual α/β-my deletion constructs. The bound and unbound fractions were then analyzed by anti-myc western blotting. The deletion of the Notch-repeat-like domains 1 and 2 (Δ433–535) or the DMAP domain (Δ698–814) did not impair the binding of the γ-subunit to the α/β-my precursor protein (Fig. 3D and E), whereas the loss of residues 431–848 prevented the interaction between α- and γ-subunits (Fig. 3C).

Next, we deleted the amino acid residues 535–698 comprising the no-similarity domain 2 located between the Notch-repeat-like and DMAP domains (Fig. 4A). The expressed α/β-my Δ535–698 precursor protein exhibits a molecular mass of 160 kDa (Fig. 4B) and lost the capability to bind to γ-GFP-bead complexes (Fig. 4C). To further define the binding site in the α-subunit, smaller deletion mutants of the no-similarity domain 2 were generated, which lack residues 535–588, 535–644 and 645–698 (Fig. 4A).

These constructs were expressed in HEK-293 cells as 180-, 165- and 175-kDa α/β-my precursor proteins, respectively, and the cleaved mature 45-kDa β-my subunits (Fig. 4B). Both N-terminal deletions of the no-similarity domain 2 (Δ535–588 and Δ535–644) bound to γ-GFP, whereas the C-terminally deleted α/β-my precursor (Δ645–698) interacted weakly with the γ-subunit (Fig. 4D-F). These data indicate that parts of amino acid residues 645–698 constitute the γ-subunit binding site of α-subunits.

When the GlcNAc-1-phosphotransferase activity was measured of the four major α-subunit mutants (Fig. 5A), the loss of residues 431–848 and 433–535 (Notch-repeat-like domains) was associated with a complete loss of GlcNAc-1-phosphotransferase activity whereas the deletion of the DMAP domain (aa 698–814) did not affect the activity at all (Fig. 5B). The deletion of the γ-subunit-interacting no-similarity domain 2 (aa 535–698),

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**Figure 2.** Binding of γ-subunits to α-subunits of GlcNAc-1-phosphotransferase. (A) Schematic representation of α-my, α/β-GFP, γ-GFP and the non-glycosylated γ-subunit (γ-N88/115Q) used in these experiments. The apparent molecular masses of the constructs (in kilodaltons) are indicated. The domain organization has been given in the legend of Figure 1A. (B and C) Extracts of HEK-293 cells overexpressing α-my (B) or γ-N88/115Q (C) were incubated with preformed γ-GFP- or α/β-GFP-GFP-Trap® bead complexes, respectively. Aliquots of the input (IN, 10%), supernatants (S, 10%), last wash fractions (W, 10%) and the bound material (B, 100%) were analyzed by mcyG, γ-GFP and γ-subunit western blotting. The positions of the molecular mass marker proteins in kilodaltons, and the glycosylated (glyc) and non-glycosylated (non-glyc) α-subunits (α-my), α/β-GFP, β-GFP and γ-subunits (γ, γ-GFP) are indicated.
however, led to a significant reduction of the GlcNAc-1-phosphotransferase activity by 30% (Fig. 5B).

In addition, CRISPR/Cas9-mediated knockout of GNPTG in human haploid fibroblasts-like HAP1 cells resulted in a significant reduction of GNPTG mRNA level (not shown) and GlcNAc-1-phosphotransferase activity by 95 and 50%, respectively, compared with control cells (Fig. 5C). These data showed that binding of γ-subunits to α-subunits is important for maximum catalytic activity of the GlcNAc-1-phosphotransferase complex.

Dimerization-independent interaction between α- and γ-subunits

The α-subunits form disulfide-linked homodimers (2). When we examined several C-terminally truncated α-subunit-GFP fusion proteins for their capability to dimerize, a miniconstruct comprising the N-terminal 430 amino acids of α-subunit (α<sub>430</sub>-GFP) was identified, which was able to form 180-kDa dimers under non-reducing conditions of SDS–PAGE (Supplementary Material, Fig. S3B, Lane 2). Under reducing conditions, only monomeric 90-kDa α<sub>430</sub>-GFP was detectable (Supplementary Material, Fig. S3C, Lane 2). The N-terminal α<sub>430</sub>-GFP construct contains five cysteine residues, C13, C23, C70, C128 and C130. C13 and C23 were excluded from being involved in interchain disulfide bridges owing to their localization in the N-terminal cytosolic and transmembrane domain, respectively. As an α<sub>430</sub>-GFP construct lacking the no-similarity domain 1 (α<sub>430</sub>Δ-GFP) harboring C128 and C130, still dimerized (Supplementary Material, Fig. S3B, Lane 4), C70 represents the most likely residue responsible for α-subunit dimerization. Substitution of C70 by serine in both α<sub>430</sub>-GFP and α<sub>430</sub>Δ-GFP prevented completely the formation of respective dimers (Supplementary Material, Fig. S3B, Lanes 3 and 5).

Next, we substituted C70 by serine (C70S) in the α/β-myc precursor protein (α/β-myc C70S; Fig. 6A) and analyzed its expression in HEK-293 cells by western blotting under non-reducing and reducing conditions. Under non-reducing conditions, the WT α/β-myc lacking aa 431–848, aa 433–535 or aa 698–814 and γ-GFP. The apparent molecular masses of the constructs (in kilodaltons) are indicated. The domain organization has been given in the legend of Figure 1A. (B) Extracts of HEK-293 cells overexpressing α/β-myc WT and mutant α/β-myc Δ431-848, α/β-myc Δ433-535 and α/β-myc Δ698-814 were resolved by SDS–PAGE and followed by anti-myc western blotting. Extracts of non-transfected (n.-t.) cells were used as negative control. (C–E) Extracts of HEK-293 cells overexpressing α/β-myc Δ431–848 (C), α/β-myc Δ433–535 (D) or α/β-myc Δ698–814 (E) were incubated with preformed γ-GFP–GFP-Trap<sup>®</sup> bead complexes. Aliquots of the input (IN, 10%), supernatants (S, 10%), last wash fractions (W, 10%) and the bound material (B, 100%) were analyzed by myc and GFP western blotting. The positions of the molecular mass marker proteins in kilodaltons and the γ-GFP, α/β-myc, and β-myc are indicated.

Figure 3. Interaction between α-subunits deletion mutants and γ-subunits of GlcNAc-1-phosphotransferase. (A) Schematic representation of α/β-myc lacking aa 431–848, aa 433–535 or aa 698–814 and γ-GFP. The apparent molecular masses of the constructs (in kilodaltons) are indicated. The domain organization has been given in the legend of Figure 1A. (B) Extracts of HEK-293 cells overexpressing α/β-myc WT and mutant α/β-myc Δ431-848, α/β-myc Δ433-535 and α/β-myc Δ698-814 were resolved by SDS–PAGE and followed by anti-myc western blotting. Extracts of non-transfected (n.-t.) cells were used as negative control. (C–E) Extracts of HEK-293 cells overexpressing α/β-myc Δ431–848 (C), α/β-myc Δ433–535 (D) or α/β-myc Δ698–814 (E) were incubated with preformed γ-GFP–GFP-Trap<sup>®</sup> bead complexes. Aliquots of the input (IN, 10%), supernatants (S, 10%), last wash fractions (W, 10%) and the bound material (B, 100%) were analyzed by myc and GFP western blotting. The positions of the molecular mass marker proteins in kilodaltons and the γ-GFP, α/β-myc, and β-myc are indicated.
The loss of α-ic shown here demonstrate that the soluble sylated region of the no-similarity domain 2 (aa 535–698) of GlcNAc-1-phosphotransferase complex binds to a glycosylated region of the α-subunits directly. This interaction is independent of the capability of α-subunits to dimerize through C70, and important for optimum GlcNAc-1-phosphotransferase activity.

To define the region in the γ-subunit that binds to no-similarity domain 2 of α-subunits, four deletion mutants, γ-GFP Δ70–129, Δ130–176, Δ177–277 and Δ239–266, were generated (Fig. 8A). Transfection of HEK-293 cells with mutant γ-GFP cDNAs resulted in the expression of 54-, 66-, 52- and 60-kDa γ-GFP fusion proteins. The isolated mutant γ-GFP-Trap® complexes were then incubated with extracts of cells transfected with α/β-myc cDNA. The most C-terminal deletion (Δ239–266) did not impair the binding of γ-subunit to α/β-myc precursor protein (Fig. 8E). The amounts of α/β-myc bound to γ-GFP mutants gradually decreases from γ-GFP Δ70–129 to Δ130–176, and being lowest in Δ177–277 (Fig. 8C, D, and E). The data suggest that an overlapping region between amino acid residues 130–238 is involved in γ-subunit interaction with no-similarity domain 2 of α-subunits.

**Discussion**

This study provides new insights into structural organization and function of the hexameric (αβγ2) GlcNAc-1-phosphotransferase complex. Our findings demonstrate that (i) γ-subunits directly bind to α-subunits, (ii) the subunit-binding site resides in the previously uncharacterized no-similarity domain 2 (which we rename as GNPTG binding (GB) domain), (iii) the loss of γ-subunit binding reduces GlcNAc-1-phosphotransferase activity, (iv) cysteine 70 is involved in disulfide-linked homodimerization of α-subunits not affecting the interaction with γ-subunits and (v) residues 130–238 of γ-subunits are involved in binding to GB domain.

(Fig. 6B) Upon reducing SDS-PAGE, immunoreactive monomeric bands of WT and mutant C70S α/β-myc precursor protein were observed (Supplementary Material, Fig. S4). Double immunofluorescence microscopy revealed that the immunoreactive α/β-myc precursor and α-subunits of the C70S mutant completely co-localized with the cis-Golgi apparatus marker protein GM130 but not with the ER marker protein disulfide isomerase (PDI; Fig. 6C) as shown for the WT protein. The substitution of C70 by serine did not affect the activity of the GlcNAc-1-phosphotransferase (Fig. 6D).

To examine whether the dimerization of α- or αβ-subunit precursor affects the interaction between α- and γ-subunits, WT α/β-myc and α/β-myc C70S mutants (Fig. 7A and B) were analyzed in γ-GFP pull-down assays. As shown in Figure 7C, the monomeric α/β-myc C70S binds effectively to γ-GFP. Together, the data shown here demonstrate that the soluble γ-subunit of the hexameric GlcNAc-1-phosphotransferase complex binds to a glycosylated region of the no-similarity domain 2 (aa 535–698) in the α-subunit. This interaction is independent of the capability of α-subunits to dimerize through C70, and important for optimum GlcNAc-1-phosphotransferase activity.

The most C-terminal deletion (Δ239–266) did not impair the binding of γ-subunit to α/β-myc precursor protein (Fig. 8E). The amounts of α/β-myc bound to γ-GFP mutants gradually decreases from γ-GFP Δ70–129 to Δ130–176, and being lowest in Δ177–277 (Fig. 8C, D, and E). The data suggest that an overlapping region between amino acid residues 130–238 is involved in γ-subunit interaction with no-similarity domain 2 of α-subunits.

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Pull-down experiments using γ-GFP-bound beads clearly showed the specific interaction with α/β-subunit precursor proteins and α-subunits, but not with β-subunits. These data supported our previous conclusions that the assembly of the hexameric, enzymatic inactive GlcNAc-1-phosphotransferase complex takes place in the ER (8). Deletion of various conserved domains in the α-subunit defined by similarities, such as stealth domain 2, Notch-repeat-like or DMAP domains or no-similarities to known proteins, further demonstrated that the residues 535–698 comprising the domain of no-similarity 2 [GB, which was also called ‘spacer region’ (16)] bear the γ-subunit binding site. Additional deletions subdividing GB domain suggest that the most C-terminal part of this domain (resides 645–698) play the major role in the subunit interaction. The lack of the GB domain impaired the efficiency of proteolytic activation by site-1 protease, which is most likely due to an increased retention in the ER resulting subsequently in a reduction of GlcNAc-1-phosphotransferase activity. On the other hand, the loss of γ-subunits led directly to reduced GlcNAc-1-phosphotransferase activity as shown for the HAP1 cells, which might be explained by decreased stability of the αβγtetramer. Four missense mutations in GB domain, G575R, R587P, A592T and T644M, identified in MLIII alpha/beta patients with slower progression of the disease, have been analyzed in more detail (16,17). These mutations resulted in variable GlcNAc-1-phosphotransferase activities (5–50% of WT controls) and could be explained by decreased mRNA stability, partial retention in the ER and impaired proteolytic activation of the α/β-subunit precursor protein in the Golgi apparatus (16,17). Interestingly, γ-subunit overexpression rescued the predominant ER localization of R587P mutant toward a predominant Golgi localization associated with a 3.5-fold increase in GlcNAc-1-phosphotransferase activity (16). Together, these data suggest two different functions of γ-subunit binding to GB domain. First, N-terminal proximity of GB to the DMAP domain described as recognition site for lysosomal enzymes (14) might explain the enhancing effect of γ-subunits on the phosphorylation of subsets of lysosomal enzymes (18). Second, the γ-subunit binding might support the correct folding of the newly synthesized α/β-subunit precursor protein also allowing the ER-Golgi transport of specific missense mutant complexes with comparable export rates.

We also demonstrated that the formation of covalently linked homodimers involving cysteine 70 in α-subunits is not required for binding to γ-subunits whereas N-glycosylation of α-subunits appears to be important for subunit interactions. The GB domain contains five potential N-glycosylation sites (N551, N607, N614, N642 and N684), but at present, it is not clear whether single or multiple N-linked oligosaccharides are required for efficient binding of γ-subunits. It is likely that the M6P receptor homology domain comprising the residues 68–170 in the γ-subunit known to bind mannosides of high mannose-type N-glycans (19) is responsible for the selective interaction with glycosylated α-subunits. This is partially supported by pull-down experiments with additional deletion mutants suggesting that residues 130–238 of γ-subunits are strongly involved in binding to α-subunits. Three of the four essential residues (R135, E153 and Y159) for recognition of mannosides (19) are present in this examined region of the γ-subunit (16). The localization of the binding region in the γ-subunit and the nature of subunit interaction (protein–carbohydrate versus protein–protein interaction) remain to be further investigated.

The present study significantly extended the knowledge on the assembly and structural as well functional interactions between subunits of the hexameric GlcNAc-1-phosphotransferase complex, a key enzyme in the biogenesis of lysosomes. Additionally, 3D crystal structure analyses of domains related to subunit interactions and catalytic functions are required to gain deeper insight into molecular pathomechanisms of mucolipidosis II and III alpha/beta.

Material and Methods

Reagents

UDP-[3H]GlcNAc was purchased from American Radiolabeled Chemicals. QAE™ Sephadex A-25 was from GE Healthcare.
Uridine 5′-diphosphate N-acetylgalcosamine (UDP-GlcNAc) sodium salt, α-methylmannoside (α-MM), adenosine 5′-triphosphate disodium salt, 4′,6-diamidino-2-phenylindol (DAPI), penicillin/streptomycin (P/S), bovine serum albumin and protease inhibitor cocktail were obtained from Sigma–Aldrich. Fetal calf serum (FCS) was from PAN Biotech. Transfection reagent JetPEI® and prestained protein ladder Pageruler™ were purchased from VWR. Dulbecco’s modified Eagle’s medium (DMEM), Iscove’s modified Dulbecco’s medium (IMDM), GlutaMAX™, Phusion® polymerase, GeneJET Plasmid Miniprep Kit and GeneJET PCR Purification Kit were from Life Technologies. Peptide N-glycosidase F (PNGase F) was from Roche. Mowiol® and Roti®quant Protein Assay was from Roth. GFP-Trap® beads were obtained from ChromoTek. Oligonucleotides used for sequencing, cloning and

Figure 6. Cysteine residue C70 is responsible for α-subunit dimerization. (A) Schematic representation of the dimeric (d) α/β-myc WT and monomeric (m) α/β-myc C70S mutant. The apparent molecular masses of the constructs (in kilodaltons) are indicated. The domain organization has been given in the legend of Figure 1A. (B) Extracts of HEK-293 cells overexpressing WT or mutant α/β-myc C70S were treated (+) or not (−) with PNGase F, resolved by SDS–PAGE under non-reducing conditions and followed by myc western blotting. Extracts of non-transfected (n.-t.) cells were used as negative control. The positions of the molecular mass marker proteins in kilodaltons and the dimeric (d) and monomeric (m) α/β-myc and β-myc are indicated. (C) HeLa cells overexpressing WT or mutant α/β-myc C70S were treated with 100 μg/ml cycloheximide for 40 min to block protein translation and release newly synthesized precursor proteins from the ER. The cells were fixed and stained with monoclonal antibodies against the α-subunit (green), the ER marker protein PDI (red) and the cis-Golgi marker protein GM130 (red). Nuclei were stained with DAPI (blue). Only merge images are shown in which yellow indicates co-localization of the antigens. Scale bar: 7 µm. (D) The activity of GlcNAc-1-phosphotransferase was measured for 60 min in extracts of HEK-293 cells overexpressing WT or mutant α/β-myc C70S (corresponding to 100-µg protein) using α-methylmannoside as acceptor. The activity of WT (corrected by the endogenous GlcNAc-1-phosphotransferase activity) was set to 100%. Data are average values of three independent experiments performed in triplicates; error bars represent SEM.

Figure 7. Dimerization of α-subunits is not required for subunit interactions. (A) Schematic representation of the dimeric (d) α/β-myc, monomeric (m) mutant C70S α/β-myc and γ-GFP. The apparent molecular masses of the constructs (in kilodaltons) are indicated. The domain organization has been given in the legend of Figure 1A. (B) Extracts of HEK-293 cells overexpressing WT or mutant C70S α/β-myc were separated by SDS–PAGE followed by anti-myc or GFP western blotting. (C) Extracts of HEK-293 cells overexpressing α/β-myc C70S were incubated with preformed γ-GFP-GFP-Trap® complexes. Aliquots of the input (IN, 10%), supernatants (S, 10%), last wash fractions (W, 10%) and the bound material (B, 100%) were analyzed by GFP and myc western blotting. The positions of the molecular mass marker proteins in kilodaltons and the γ-GFP, α/β-myc and β-myc are indicated.
mutagenesis were synthesized by MWG Eurofins. The expression plasmids pcDNA3.1D-TOPO® and pEGFP_N1 were from Life Technologies and Clontech, respectively.

**Antibodies**

Monoclonal antibodies against HA and GFP were purchased from Roche. Monoclonal antibodies against myc, PDI and GM130 were from Cell Signalling, Biomol and BD Biosciences, respectively. The monoclonal rat antibody against the human α-subunit and the polyclonal rabbit antibody against the human γ-subunit of the GlcNAc-1-phosphotransferase were described previously (15,20). Secondary antibodies conjugated to horseradish peroxidase, anti-mouse Alexa Fluor® 546 and anti-rat Alexa Fluor® 488 were purchased from Dianova and Life Technologies, respectively.

**Generation of cDNA constructs**

Generation of the expression constructs for C-terminally myc-tagged full-length α/β-subunit precursor (α/β-myc) and C-terminally myc-tagged α-subunit (α-myc) and N-terminally HA-tagged β-subunit (HA-β) of the GlcNAc-1-phosphotransferase using the expression vector pcDNA3.1D-TOPO® was previously described (9). To delete the amino acids 431–848, 433–535, 698–814, 535–588, 535–644, 535–698 or 645–698 from the α/β-myc construct, megaprimers (Supplementary Material, Table S1, #5–18) were used. The primers were designed to have a complementary sequence flanking the deletion. After annealing of the megaprimers, the regions to be deleted loop out and are excluded from the PCR [21]. For the generation of C-terminally GFP-tagged α/β-subunit precursor construct (α/β-GFP), megaprimers containing the GFP sequence, and short forward and reverse primers, which anneal both to the template sequences of the vector pEGFP_N1 and the α/β-myc construct were used (Supplementary Material, Table S1, #19 and 34). For the construct α430-GFP, the N-terminal part of the γ-subunit was amplified by PCR. For the generation of α430/α266-GFP lacking aa 87–321, overlap extension PCR was used (Supplementary Material, Table S1, #19–24). PCR products were cloned into the expression vector pEGFP_N1 using restriction enzymes BamHI and XhoI. The mutation C70S was inserted into α/β-myc, α430-GFP and α430/α266-GFP constructs by site-directed mutagenesis using mutagenic primers and Phusion® polymerase. The two complementary oligonucleotides (Supplementary Material, Table S1, #1 and 2) with the desired mutation were designed using the web-based program PrimerX (www.bioinformatics.org/primerx). The megaprimers were used to amplify the amino acids 70–129, 130–176, 177–277 and 239–266 from the GFP-tagged γ-subunit (Supplementary Material, Table S1, #25–32). The generation of non-tagged α-subunit, glycosylation-mutant γ-N88/115Q and C-terminally GFP-tagged γ-subunit (γ-GFP) were described previously (8). All plasmid DNAs were commercially sequenced (SeqLab, Göttingen, Germany).

**Cell culture and transfections**

HEK-293 and HeLa cells were maintained in DMEM supplemented with 10% FCS, GlutaMAX™ and F/S at 37°C and 5% CO₂. Cells grown on 6-cm plates or on glass cover slips were transiently
transfected with cDNA constructs using JetPEI® reagent according to the manufacturer instructions. Thirty hours after transfection, the cells were used for further analysis.

Human haploid HAP1 cells lacking GNPTG (ΔGNPTG; clone HAP1_GNPTG_23585-05) were commercially generated by Horizon Genomics using the CRISPR/Cas9 system. Parental control and ΔGNPTG-HAP1 cells were cultured in IMDM supplemented with 10% FCS, Glutamax™ and P/S at 37°C and 5% CO₂.

GFP-Trap® beads pull-down experiments
HEK-293 cells transiently transfected with GFP or GFP fusion constructs were lysed in 10 mM Tris/HCl buffer pH 7.4 containing 150 mM NaCl, 0.5 mM EDTA, 0.1% Nonidet P40 and protease inhibitors and centrifuged at 16,000×g for 10 min at 4°C. Cell extracts corresponding to 500 µg protein were incubated with 25 µl equilibrated GFP-Trap® beads for 2 h at 4°C. After removal of unbound material by centrifugation (17,200×g for 10 min at 4°C), the supernatant was removed, and the beads were washed three times each with 1 ml lysis buffer lacking Nonidet P40. The bound material was solubilized in reducing SDS/PAGE buffer. Ten percent of the input (IN), the non-bound supernatant (S) and the last wash fraction (W) as well as the total bound fraction were separated by SDS–PAGE (10% acrylamide) and processed for western blotting.

Other methods
Confocal microscopy of transfected cells, preparation of cell extracts, measurements of GlcNAc-1-phosphotransferase activity, enzymatic deglycosylation of proteins and SDS–PAGE followed by western blot analysis were performed as described recently (17,20).

Statistical analysis
Results are expressed as means ± SEM. Differences between mean values are determined using the Student’s t-test. P-values of <0.005 were considered significant.

Supplementary Material
Supplementary Material is available at HMG online.

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