Transport of the GlcNAc-1-phosphotransferase α/β-Subunit Precursor Protein to the Golgi Apparatus Requires a Combinatorial Sorting Motif

Received for publication, August 3, 2012, and in revised form, November 22, 2012 Published, JBC Papers in Press, November 28, 2012, DOI 10.1074/jbc.M112.407676

Mine Franke, Thomas Braulke, and Stephan Storch

From the Department of Biochemistry, Children’s Hospital, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany

Background: Golgi-resident GlcNAc-1-phosphotransferase is the key enzyme for biosynthesis of mannose 6-phosphate recognition marker.

Results: Identification of a combinatorial ER export motif in the N- and C-terminal cytosolic domains of the GlcNAc-1-phosphotransferase α/β-subunit precursor protein.

Conclusion: COPII-dependent ER export of the phosphotransferase precursor is mediated by dileucine/dibasic sorting motifs.

Significance: Novel insights into ER sorting of type III membrane proteins are discussed.

The Golgi-resident N-acetylgalactosamine-1-phosphotransferase (PT) complex is composed of two α-, β-, and γ-subunits and represents the key enzyme for the biosynthesis of mannose 6-phosphate recognition marker on soluble lysosomal proteins. Mutations in the PT complex cause the lysosomal storage diseases mucolipidosis II and III. A prerequisite for the enzymatic activity is the site-1 protease-mediated cleavage of the PT α/β-subunit precursor protein in the Golgi apparatus. Here, we have investigated structural requirements of the PT α/β-subunit precursor protein for its efficient export from the endoplasmic reticulum (ER). Both wild-type and a cleavage-resistant type III membrane PT α/β-subunit precursor protein are exported whereas coexpressed separate α- and β-subunits failed to reach the cis-Golgi compartment. Mutational analyses revealed combinatorial, non-exchangeable dileucine and dibasic motifs located in a defined sequence context in the cytosolic N- and C-terminal domains that are required for efficient ER exit and subsequent proteolytic activation of the α/β-subunit precursor protein in the Golgi. In the presence of a dominant negative Sar1 mutant the ER exit of the PT α/β-subunit precursor protein is inhibited indicating its transport in coat protein complex II-coated vesicles. Expression studies of missense mutations identified in mucolipidosis III patients that alter amino acids in the N- and C-terminal domains demonstrated that the substitution of a lysine residue in close proximity to the dileucine sorting motif impaired ER-Golgi transport and subsequent activation of the PT α/β-subunit precursor protein. The data suggest that the oligomeric type III membrane protein PT complex requires a combinatorial sorting motif that forms a tertiary epitope to be recognized by distinct sites within the coat protein complex II machinery.

N-Acetylgalactosamine (GlcNAc)2-1-phosphotransferase (PT) is a Golgi-resident hexameric complex composed of three subunits, α, β, and γ. The bovine 540-kDa PT complex was proposed to exist in an αβγγ configuration containing disulfide-linked αγ- and γγ-dimers (1, 2). The human PT α/β-subunit precursor protein (NP_077288) and the γ-subunit protein (NP_115909) are encoded by the GNPTAB and the GNPTG genes, respectively (3–5). The PT α/β-subunit precursor protein represents a type III membrane protein of 1256 amino acids with the N and C termini facing the cytosol (3). The cleavage of the PT α/β-subunit precursor protein, which occurs between residues Lys-928 and Asp-929 is catalyzed by the site-1 protease localized in the Golgi apparatus and results in the generation of catalytically active α- and β-subunits (4, 6). The PT α/β-subunits harbor the binding site for the substrate UDP-GlcNAc, recognize a common protein determinant of acid hydrolases and contain the catalytic activity (7). The PT γ-subunit is synthesized as a soluble glycoprotein of 305 amino acids of unknown function (5). Mutations in the GNPTAB gene lead to mucolipidosis (ML) II α/β (MIM ID 252500), and ML III α/β (MIM ID 252600), whereas mutations in the GNPTG gene result in ML III γ (MIM ID 252605; reviewed in Ref. 8). The consequences of sequence alterations in the PT α/β-subunit precursor for stability, subunit assembly, binding of substrates and lysosomal enzymes, intracellular transport between the endoplasmic reticulum (ER) and Golgi apparatus, proteolytic cleavage, and posttranslational modifications of the PT complex are unknown.

Sorting signals present in the cytoplasmic domains of membrane proteins have been reported to mediate the efficient anterograde transport from the ER to the Golgi apparatus in coat protein complex II (COPII)-coated vesicles (9). Two main

*This work was supported by Deutsche Forschungsgemeinschaft Grants STO 761/2-1 and GRK1459.

□ This article contains supplemental Table S1 and Fig. S1.

1 To whom correspondence should be addressed: Dept. of Biochemistry, Children’s Hospital, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, Bldg. N27, 20246 Hamburg, Germany. Tel.: 49-40-7410-51967; Fax: 49-40-741058504; E-mail: storch@uke.uni-hamburg.de.

2 The abbreviations used are: GlcNAc, N-acetylgalactosamine; PT, GlcNAc-1-phosphotransferase; ML, mucolipidosis; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; ER-Ret, ER retention signal; COPII, coat protein complex II; PDI, protein disulfide isomerase; PNGase F, peptide/N-glycosidase F; endo H, endoglycosidase H; C-tail, carboxyterminal tail; EGFP, enhanced GFP.
classes of sorting signals have been characterized in type I and III membrane proteins, which comprise diacidic (DE)X(DE) motifs (where X is any amino acid), and short hydrophobic motifs, such as LL, II, FY, YYM, and FF (10). In addition, dibasic (RK)X(RK) motifs have been identified mediating the ER export of Golgi resident type II membrane glycosyltransferases (11). In multispanning membrane proteins and oligomeric complexes, multiple sorting signals promote both ER exit and ER retention of unfolded or unassembled subunits of protein oligomers (9, 12). Export signals are recognized by the COP II prebudding protein complex that comprises the small GTPase Sar1 and the Sec23-Sec24 heterodimer (13, 14).

In the present study, we have analyzed the structural requirements for the transport of the PT α/β-subunit precursor protein from the ER to the Golgi apparatus. The acquisition of endoglycosidase H-resistance, site-1 protease-mediated cleavage, and co-localization with Golgi marker proteins were used as criteria for the arrival of the PT α/β-subunit precursor protein in the Golgi apparatus and allowed the identification of required signal structures. The data demonstrate that the PT α/β-subunit precursor protein exhibits combinatorial sorting motifs composed of a dominant dileucine and a dibasic RIR motif in the N- and C-terminal cytosolic domains, respectively, required for efficient ER export in COP II-coated vesicles.

**EXPERIMENTAL PROCEDURES**

**Reagents**—FastDigest™ restriction enzymes, alkaline phosphatase, T4 DNA ligase, GeneJET™ plasmid miniprep kit, GeneJET™ gel extraction kit, GeneJET™ PCR purification kit, dNTPs, Phusion® polymerase, PageRuler, enhanced chemoluminescent (ECL) reagent, and bovine serum albumin (BSA) were obtained from Thermo Fisher Scientific (Schwerte, Germany), and a Plasmid Midi kit was obtained from Qiagen (Hilden, Germany). Oligonucleotides used for cloning, sequencing, and mutagenesis were purchased from MWG Biotech (Munich, Germany). Peptide N-glycosidase F (PNGase F) and PVDF membranes were from Roche Applied Science, and endoglycosidase H (endo H) was from New England Biolabs (Frankfurt, Germany). Media for cultivating *Escherichia coli*, carbenicillin, kanamycin, dithiothreitol, and Roti® quant microassay kit were from Roth (Karlsruhe, Germany). 4′,6′-diamidino-2-phenylindol (DAPI), cytochrome oxidase inhibitor mixture, saponin, and other common laboratory reagents were purchased from Sigma-Aldrich. Jet PEI® was from Polyclplus-Transfection (Ilkirch Cedex, France), Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, and Lipofectamine™ 2000 were from Invitrogen. Fetal calf serum (FCS) was purchased from PAA Laboratories (Pasching, Austria).

**Antibodies**—The following antibodies were used for Western blot analyses: polyclonal rabbit anti-red fluorescent protein (1:1,100), monoclonal anti-α-tubulin (1:10,000, Sigma-Aldrich), monoclonal anti-Myc (1:5,000, clone 9B11, Cell Signaling), and monoclonal rat anti-hemagglutinin A (HA) antibody (1:5,000, clone 3F10, Roche Applied Science). Secondary antibodies used for immunoblotting were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000), goat anti-rat IgG (1:5,000), and goat anti-mouse IgG (1:5,000) from Jackson Immunoresearch Laboratories, Inc. For double immunofluorescence microscopy, polyclonal rabbit anti-Myc (1:100, Sigma-Aldrich), monoclonal anti-protein disulfide isomerase (PDI; clone 1D3, 1:500), monoclonal anti-ERGIC-53 (clone G1/93; 1:100, Enzo Life Sciences, Lörrach, Germany), monoclonal anti-GM130 (1:100; BD Biosciences), Alexa Fluor® goat anti-rabbit 488, and Alexa Fluor® goat anti-mouse 594 (1:1,000; Invitrogen) antibodies were used.

**Vector Constructs**—Expression vector pEGFP-N1 was purchased from Clontech (Palo Alto, CA). All oligonucleotide primers used for cloning, site-directed mutagenesis, and sequencing are listed in [supplemental Table S1](#). The human PT α/β-subunit precursor cDNA sequence (GenBank™ accession no. NM_024312) was described previously (3) and amplified by PCR using primers 1F/R. The resulting PCR product was cloned into the eukaryotic expression vector pcDNA™3.1/D/V5-His-TOPO® (Invitrogen), resulting in the PT α/β-Myc cDNA expression construct. The PT α-subunit cDNA was fused to a C-terminal Myc epitope tag (PT α-Myc) by amplifying the PT α/β-subunit precursor cDNA template with primers 2F/R. The resulting PCR product was cloned into pcDNA™3.1/D/V5-His-TOPO®. The PT β-cDNA was fused to the N-terminal signal murine Ig κ-chain leader signal sequence and an HA epitope tag (HA-PT β) by subcloning a PCR product amplified with primers 3F/R into the SacII and Sall restriction sites of vector pDisplay (Invitrogen). Mutations were introduced by PCR using Phusion® polymerase (Thermo Fisher Scientific) and a modified site-directed mutagenesis protocol (15). Double, triple, and quadrupole mutations were introduced by subsequent site-directed mutagenesis reactions with single primer pairs (4F/R to 10F/R) and DNA templates carrying single, double, and triple mutations, respectively. Mutant PT α/β-Myc K4Q, K1236M, and R925A cDNA constructs were generated by site-directed mutagenesis using primers 11F/R, 12F/R, and 13F/R, respectively, with expression vector pcDNA™3.1/D/V5-His-TOPO® PT α/β-Myc as the template. The C-terminal amino acids of the human α2C-adrenergic receptor (KHILFRRRRRGRFQR) were fused to the Myc epitope of the human PT α/β-subunit precursor by PCR using primers 14F/R and pFROG-mLIMP II-ER as DNA template (16, 17). The resulting PCR product was used as a megaprimer with pcDNA3.1.TOPO® PT α/β-Myc as a template (18). A full-length IMAGE clone (IRAup969C1220D containing the cDNA of human Sar1A (GenBank™ accession no. NM_001142648) was purchased from imaGenes (Berlin, Germany). A 660-bp PCR product was amplification using primers 15F/R using the Sar1 cDNA clone as a template and cloned into pcDNA™3.1/D/V5-His-TOPO® expression vector. The dominant negative mutation H79G (19) was introduced into the Sar1 cDNA by site-directed mutagenesis using oligonucleotides 16F/R. Wild-type and mutant Sar1 H79G fused to an mCherry tag were cloned by amplifying the Cherry cDNA sequence using primers 17F/R and the pCS2 mCherry vector as DNA template (20). The resulting PCR product was used as megaprimer in a modified site-directed mutagenesis reaction with pcDNA3.1.TOPO® Sar1 wild-type and pcDNA3.1.TOPO® Sar1 H79G, respectively, as DNA templates. Sequencing of all constructs using primers 18F-26F was performed by SeqLab (Göttingen, Germany).
Cell Culture—COS-7 and HeLa cells were cultivated in DMEM containing 10% FCS supplemented with glutamine and penicillin/streptomycin. Transfection of cells was performed with Jet PEI® and Lipofectamine™ 2000 (Invitrogen). Cells were analyzed 24 to 36 h hours after the start of transfection.

Western Blotting—Prior to harvesting, COS-7 cells were incubated in the presence of 100 μg/ml cycloheximide for 4 h. Cells were solubilized on ice in lysis buffer containing 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate supplemented with protease inhibitors and centrifuged at 12,000 × g at 4 °C for 10 min. Protein concentrations of the supernatants were determined using the Roti® quant microassay kit with BSA as standard. Cell extracts were separated by SDS-PAGE, blotted onto PVDF membranes, blocked in 25 mM Tris-buffered saline, pH 7.4, and incubated in the presence of 100 μg/ml cycloheximide for 4 h.

Evaluation of protein loading of the gels was verified by α-tubulin Western blotting. Blots were imaged on a Molecular Imager (Model Chemi Doc XRS, Bio-Rad). Densitometric analysis was performed using Image lab software (Bio-Rad). Statistical significance was evaluated with a two-tailed, unpaired t test with Graph Pad Prism (Graph Pad Software).

Enzymatic Deglycosylation of Proteins—For enzymatic deglycosylation of proteins total cell extracts were incubated with PNGase F or endo H for 1 h at 37 °C as described previously (21).

Confocal Immunofluorescence Microscopy—COS-7 cells grown on glass coverslips were transfected with Lipofectamine™ 2000 and incubated with cycloheximide (100 μg/ml) in full medium for 2 h prior to fixation. Cells were fixed in 4% paraformaldehyde and permeabilized with 10 mM phosphate-buffered saline, pH 7.4, containing 0.1% saponin. After incubation with primary and Alexa Fluor®-coupled secondary antibodies and staining of nuclei with DAPI, coverslips were sealed in mounting medium (DAKO, Glostrup, Denmark). Double immunofluorescence microscopy was performed as described previously (22). Images were taken with a Leica digital scanning confocal microscope (Leica DMIRE2, 63× magnification), and merge of images was performed using Adobe Photoshop software.

RESULTS

Both Uncleaved and Cleaved Forms of the PT α/β-Subunit Precursor Are Localized in the Golgi Apparatus—To analyze the transport of the PT α/β-subunit precursor, COS-7 cells were transfected with a cDNA coding for the PT α/β-Myc subunit precursor (PT α/β-Myc) fusion protein. Western blotting revealed the presence of 190-kDa PT α/β-Myc subunit precursor and 45-kDa β-Myc polypeptides, that were not detectable in extracts of pEGFP-transfected control cells (Fig. 1A and B, lanes 1 and 2). After treatment of cell extracts with endo H and PNGase F, the molecular masses of the PT α/β-Myc subunit precursor shifted to 170-kDa, indicating the presence of endo H-sensitive N-linked high mannose-type oligosaccharides (Fig. 1B, lanes 3 and 4). The molecular mass of the PT β-Myc subunit shifted to a doublet of 43/42-kDa or 40-kDa after treatment with endo H and PNGase F, respectively, indicating the presence of endo H-resistant complex-type N-glycans (Fig. 1B, lanes 3 and 4). As control for PT α/β-subunit precursor protein cleavage in the Golgi apparatus, COS-7 cells were transfected with a cDNA coding for PT α/β-Myc fused to a C-terminal ER retention signal of the human α2C-adrenergic receptor (KHLFRRRRRGGFRQ, PT α/β-Myc-ER-RET, Fig. 1A). In cells expressing PT α/β-Myc-ER-RET, only the 190-kDa subunit precursor containing endo H-sensitive N-linked glycans could be detected, but not the cleaved PT β-subunit (Fig. 1B, lanes 5–7). To confirm the data, localization of Myc-reactive wild-type and ER-retained PT α/β-Myc subunit precursors was studied by double immunofluorescence microscopy. Wild-type PT α/β-Myc subunit precursor protein and cleaved PT β-Myc subunit co-localized mainly with the ER-Golgi intermediate compartment (ERGIC) marker protein ERGIC-53 (Fig. 1C, panel f) and the cis-Golgi marker protein GM130 (Fig. 1C, c), but not with the ER marker protein disulfide isomerase (PDI, Fig. 1C, panel i). In contrast, no co-localization of PT α/β-Myc-ER-RET with the ERGIC and Golgi marker proteins was observed (Fig. 1C, panels l and o), but complete overlapping localization with the ER marker protein (Fig. 1C, r). To further demonstrate that the uncleaved full-length PT α/β-subunit precursor protein is localized at steady state in the Golgi apparatus, the arginine 925 residue was substituted by alanine, which resulted in an uncleavable PT α/β-subunit precursor protein (PT α/β-Myc R925A, Fig. 2A, lane 6). This mutant is expressed as 230- and 190-kDa immunoreactive polypeptide most likely representing complex-type endo H-resistant and high mannose-type endo H-sensitive glycosylated forms, respectively (Fig. 2A, lane 5–7). The fusion with the ER retention signal of the α2C-adrenergic receptor to the R925A mutant resulted in endo H-sensitive glycans only (Fig. 2A, lane 9) demonstrating the potency of the signal in retention of type III membrane proteins in the ER. Double immunofluorescence microscopy of transfected COS-7 cells showed that PT α/β-Myc R925A co-localized mainly with the cis-Golgi marker protein GM130 (Fig. 2B, panels c–c), whereas no co-localization was detectable with the ER-resident PDI protein (Fig. 2B, panels d–f). As expected, localization of PT α/β-Myc R925A ER-RET overlapped mainly with PDI (Fig. 2B, panels g–i), but not with GM130 indicating that the export from the ER failed (Fig. 2B, panels h–l). These results suggest that both uncleaved and cleaved forms of the PT α/β-subunit precursor are localized in the Golgi apparatus at steady state. Retention of the PT α/β-subunit precursor protein in the ER prevents proteolytic cleavage into the PT α- and β-subunits.

Transport of the PT α/β-Subunit Precursor Protein to the Golgi Apparatus—To monitor transport of the PT α/β-subunit precursor protein from the ER to the Golgi apparatus the fraction of cleaved, endo H-resistant PT β-subunit and its degree of co-localization with the cis-Golgi marker protein GM130 were evaluated. To examine whether only the PT α/β-subunit precursor protein exits the ER, the single PT α- and β-subunits were expressed separately or in combination in COS-7 cells. The PT α-Myc construct is expressed as polypeptide with an apparent molecular mass of 130-kDa (Fig. 3A, lane 4). Both after endo H and PNGase F treatment, the molecular mass of
The Triple Arginine Motif in the C-terminal Cytosolic Domain of the PT β-Subunit Is Not a Functional ER Retention Signal—For a number of multisubunit proteins, triple arginine-based ER localization signals have been identified in the C-terminal cytoplasmic domain, which conform to the consensus sequence (ψψψ/R)XRXR, where ψψψ are bulky hydrophobic or aromatic amino acids and X is any residue (12). The cytoplasmic domain of the PT β-subunit also contains a triple arginine motif (1242RRR1244). To examine whether this motif functions as ER retention signal of the PT β-subunit, the triple arginine residues were substituted by alanines (HA-PT β-RRR→AAA). To exclude additional retention signals, the complete cytoplasmic tail of the PT β-subunit (21 amino acids) was deleted (HA-PT β ΔCT). The constructs were expressed in COS-7 cells, and the cell extracts were treated or not with endo H or PNGase F. In COS-7 cells expressing HA-PT β ΔCT, an immunoreactive band with slightly higher electrophoretic mobility compared with the wild-type or the mutant HA-PT β RRR→AAA was detected, which is due to the deletion of C-terminal domain (Fig. 4, lane 8 versus 2 and 5). Both wild-type and mutant PT β shifted to 100-kDa, indicating the presence of endo H-sensitive N-linked oligosaccharides (Fig. 3A, lanes 4–6). In COS-7 cells transfected with the HA-PT β-cDNA construct, a 45-kDa immunoreactive polypeptide was detected. In contrast to the PT β-subunit generated after cleavage of the PT α/β-subunit precursor protein, the separately expressed HA-PT β cDNA is completely endo H-sensitive, suggesting that this subunit cannot exit the ER (Fig. 2A, lane 2 versus 8). To rule out that the retention of the isolated PT β-subunit is due to the lack of the α-subunit, the individual subunits were expressed together. The co-expression of PT α-Myc and HA-PT β cDNA constructs did not affect the endo H sensitivity of the PT β-subunit (Fig. 3A, lanes 10–12), indicating that ER exit requires the PT α/β-subunit precursor protein, whereas the separated PT-subunits alone or in combination are not sufficient to reach the Golgi apparatus. Double immunofluorescence microscopy confirmed that the isolated PT α- and β-subunits co-localized mainly with the ER-resident PDI (Fig. 3B, panels f and l) and failed to co-localize with the Golgi marker protein GM130 (Fig. 2B, panels c and l).
forms contain endo H-sensitive N-glycans only (Fig. 4, lanes 6 and 9), indicating their retention in the ER and the absence of ER retention signals in the cytoplasmic tail of the PT β-subunit.

**ER Export Motifs of the PT α/β-Subunit Precursor Protein**—To identify signal structures within the cytoplasmic domains of the PT α/β-subunit precursor protein that mediate its transport from the ER to the Golgi apparatus, the sequences of the N- and C-terminal cytosolic domains were compared with consensus sequences of known ER export motifs (9). Four potential highly conserved ER export motifs were found in the cytoplasmic domains of the PT α/β-subunit precursor protein: a dileucine motif (5LL6) in the N-terminal domain and three dibasic (RK)X(RK)-based motifs (1236KRK1238, 1242RRR1244, and 1253RIR1255) in the C-terminal domain (Fig. 5A). To analyze the role of these potential ER export motifs, alanine substitutions of critical residues alone or in combination were introduced into the PT α/β-subunit precursor cDNA followed by transient expression in COS-7 cells. Cell extracts were analyzed by Myc-Western blotting and the presence of cleaved PT β-Myc was used as indicator of ER exit (Fig. 5B).

Replacement of the N-terminal dileucine motif 5LL6 with alanine residues resulted in a significant reduction of the PT α/β-subunit precursor protein cleavage (Fig. 5B, lanes 2 and 4). Densitometric evaluation of intensities of immunoreactive bands (ratio: PT β-Myc 5LL6/PT β-Myc) detectable in cells expressing PT α/β-Myc 5LL6 → AA mutant showed that the formation of the β-subunit was reduced by 46 ± 18% compared with the wild-type construct. Alanine substitutions of the individual C-terminal 1236KRK1238, 1242RRR1244, and 1253RIR1255 motifs revealed that the relative amounts of cleaved PT β-subunits were similar (Fig. 5B, lanes 5–7, Fig. 5C). When the N-terminal dileucine motif was substituted by alanine residues in combination with one of the C-terminal (RK)X(RK)-based motifs (Fig. 5B, lanes 8–10), the double mutant 5LL6/
1253RIR1255 showed the strongest inhibitory effect on the cleavage of the PTβ/H9251/H9252-subunit precursor protein (80% compared with the wild-type precursor protein), which was comparable with the PTβ/H9251/H9252-Myc-ER-Ret mutant (Fig. 5B, lanes 3 and 10). Combined alanine-substitution of each two C-terminal dibasic motifs resulted in a decrease in the amount of cleaved PTβ-subunit by 13% (1236KRK1238/1242RRR1244) and 34% (1236KRK1238/1253RIR1255) and 52% (1242RRR1244/1253RIR1255), respectively, compared with the expressed wild-type PTβ/H9251/H9252- subunit precursor protein (Fig. 5B, lanes 11–13 and supplemental Fig. S1). Of note, expression of double motif mutants 1236KRK1238/1253RIR1255 and 1242RRR1244/1253RIR1255 resulted in cleaved PTβ-subunits exhibiting slightly increased molecular masses (Fig. 5B, lanes 12 and 13). The reason for the altered electrophoretic mobilities is unclear. In extracts of cells expressing PT α/β-Myc with combined substitution of the

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**FIGURE 3.** The isolated PT α- and β-subunits are retained in the ER. A, COS-7 cells were transfected with PT α/β-Myc subunit precursor, PT α-Myc subunit, HA-PT β subunit cDNA constructs, or co-transfected with PT α-Myc and HA-PT β cDNAs. Total cell extracts (100 μg) were incubated in the absence (lanes 1, 4, 7, and 10) or presence (+) of endo H (lanes 2, 5, 8, and 11) and PNGase F (lanes 3, 6, 9, and 12), separated by SDS-PAGE and analyzed by Myc- or HA-Western blotting (WB). Equal loading of the gel was verified by α-tubulin Western blotting. The positions of the molecular mass marker proteins, the α/β-subunit precursor, and the individual PT subunits are indicated. B, COS-7 cells were transfected with PT α-Myc (panels a–f) and HA-PT β cDNA constructs (panels g–l), fixed after 24 h, and incubated with antibodies against Myc (panels a and d; green), HA (panels g and j; green), the cis-Golgi marker protein GM130 (panels b and h; red), and the ER marker protein PDI (panels e and k; red). Nuclei were visualized by DAPI (blue). In the merged images (panels c, f, i, and l), yellow indicates co-localization. Scale bars, 10 μm.

**FIGURE 4.** The C-terminal domain of the isolated PT β-subunit lacks an ER retention signal. COS-7 cells were transfected with cDNAs coding for EGFP control, wild-type, and mutant HA-PT β-subunit with alanine substitution of the potential ER retention motif (HA-PT β RRR→AAA) or deletion of the entire C-terminal tail (HA-PT β ΔCT). Total cell extracts were prepared, incubated in the absence (−) or presence (+) of endo H or PNGase F and analyzed by HA Western blotting (WB). After electrotransfer of the proteins, PVDF membranes were probed with antibodies against HA followed by incubation with HRP-coupled secondary antibodies and ECL analysis. Positions of the N-glycosylated PT β-subunit and of the molecular mass marker proteins are indicated.
dileucine-based motif and two or all C-terminal (RK)(RK)-based motifs the decrease in cleaved PT subunit was comparable with 5LL6/1253RIR1255 or the ER-Ret mutants (Fig. 5B, lanes 14–18, and supplemental Fig. S1). Double immunofluorescence microscopy analyses revealed that PT subunit mainly co-localized with the cis-Golgi marker protein GM130 (Fig. 6, a3, c3, e3, and g3) but failed to co-distribute with the ER marker protein PDI (Fig. 6, b3, d3, f3, and h3). Combined mutation of the 5LL6 and 1253RIR1255 motifs (LL→AA/RIR→AAA) led to retention of the PT subunit precursor protein in the ER as shown by the overlapping distribution with PDI (Fig. 6, j3). A minor fraction still co-localized with the cis-Golgi marker protein GM130 (Fig. 6, i3).

ER Exit Depends on Two Distinct Signals—Next, we examined whether the efficient ER export of PT subunit precursor protein requires the presence of two distinct signals or two identical sorting signals in two different positions in the N and C terminus. Replacement of the N-terminal 5LL6 motif by RIR or RR residues (RIR/RIR or RR/RIR) led to the PT subunit precursor protein with two basic sorting motifs in the N- and C-terminal domain. These mutations inhibited the cleavage of the PT subunit precursor protein similarly to the PT subunit precursor protein with two basic sorting motifs in the N- and C-terminal domain. Combined mutation of the 5LL6 and 1253RIR1255 motifs (LL→AA/RIR→AAA) or the dibasic motif mutants (1236KRK1238AAA, 1242RRR1244AAA, 1253RIR1255AAA) led to retention of the PT subunit precursor protein in the ER as shown by the overlapping distribution with PDI (Fig. 6, j3). A minor fraction still co-localized with the cis-Golgi marker protein GM130 (Fig. 6, i3).

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ER Exit Depends on Two Distinct Signals—Next, we examined whether the efficient ER export of PT subunit precursor protein requires the presence of two distinct signals or two identical sorting signals in two different positions in the N and C terminus. Replacement of the N-terminal 5LL6 motif by RIR or RR residues (RIR/RIR or RR/RIR) led to the PT subunit precursor protein with two basic sorting motifs in the N- and C-terminal domain. These mutations inhibited the cleavage of the PT subunit precursor protein similarly to the PT subunit precursor protein with two basic sorting motifs in the N- and C-terminal domain. Combined mutation of the 5LL6 and 1253RIR1255 motifs (LL→AA/RIR→AAA) led to retention of the PT subunit precursor protein in the ER as shown by the overlapping distribution with PDI (Fig. 6, j3). A minor fraction still co-localized with the cis-Golgi marker protein GM130 (Fig. 6, i3).
α/β-Myc 5LL<sup>6</sup>→AA mutant (Fig. 7B, lanes 7 and 8 versus lane 4). Furthermore, substitution of the N-terminal 5LL<sup>6</sup> motif by dibasic-based motifs RIR or RR in combination with alanine substitution of the C-terminal 1254/RR<sup>1255</sup> motif (RR/AAA or RR/AAA) prevented the α/β-subunit precursor protein from reaching the Golgi apparatus for cleavage (Fig. 7B, lanes 10 and 11). Surprisingly, two dileucine sorting motifs in the N- and C-terminal domain of the PT α/β-subunit precursor protein (LL/LL) strongly reduced the proteolytic processing to the mature PT β-subunit (Fig. 7B, lane 9). Fig. 7B also shows that the transfer of the dileucine motif to the C-terminal domain replacing the dibasic-based motif 1254/RR<sup>1255</sup> in combination with the substitution of the N-terminal dileucine motif (AA/LL), blocked the ER exit and the subsequent proteolytic cleavage to mature PT β-subunit (Fig. 7B, lane 12). From these data, we conclude that two identical dileucine or dibasic-based motifs in two different positions, namely in the N- and C-terminal domain, are not sufficient to mediate efficient ER exit of the PT α/β-subunit precursor protein. Furthermore, single dileucine or dibasic sorting information contained in the N and C terminus, respectively, was not exchangeable and indicate that two distinct sorting motifs in the context of two different positions are required for ER exit and site-1 protease-mediated cleavage of the PT α/β-subunit precursor protein in the Golgi apparatus.

Patient Mutation K4Q Impairs the ER Export of the PT α/β-Subunit Precursor—To analyze whether mutations in cytosolic domains found in MLIII α/β patients with milder phenotype affect ER export and proteolytic cleavage of the PT α/β-subunit precursor protein, we introduced missense mutations K4Q (23) and K1236M (24) into the PT α/β-Myc cDNA. COS-7 cells were transfected with wild-type and mutant PT α/β-Myc K4Q or K1236M, and cell extracts were analyzed by Western blotting (Fig. 8A). In cells transfected with the mutant PT α/β-Myc K4Q reduced levels of the 45-kDa PT β-subunit were observed compared with the wild-type PT α/β-subunit precursor protein, whereas the proteolytic cleavage of PT α/β-Myc K1236M was not affected (Fig. 8A, lanes 4 and 5). GFP and the ER retention mutant were used as controls (Fig. 8A, lanes 1 and 3). Densitometric analyses of the blots revealed statistically significant decreases in the amount of cleaved PT β-subunit by 70% in PT α/β-K4Q precursor protein expressing cells (Fig. 8B). Double immunofluorescence analyses of COS-7 cells transfected with PT α/β-Myc K4Q cDNA showed that the K4Q mutant co-localized both with the ER marker PDI and the cis-Golgi marker protein GM130 (Fig. 8C, panels a–f). Mutant PT α/β-Myc K1236M co-localized mainly with the cis-Golgi marker protein GM130 (Fig. 8C, panels g–i). The results demonstrate that MLIII patient mutation K4Q in the proximity of the 5LL<sup>6</sup> sorting motif impairs the efficient exit of the PT α/β-subunit precursor protein from the ER and subsequently the proteolytic cleavage into the catalytically active PT α- and β-subunits in the Golgi apparatus.

Sar1-dependent ER Export of the PT α/β-Subunit Precursor—For a number of type I, type II, and type III membrane proteins sorting from the ER to the Golgi apparatus is dependent on the small GTPase Sar1 (25, 26). We analyzed the potential role of Sar1 for the transport of the PT α/β-subunit precursor protein from the ER to the Golgi apparatus in cells co-expressing a GTP-restricted, dominant-negative Sar1H79G mutant (19). COS-7 cells were either transfected with PT α/β-Myc cDNA alone or co-transfected with PT α/β-Myc and wild-type or mutant Sar1H79G mCherry (Fig. 9A). The EGFP and PT α/β-Myc-ER-Ret cDNA constructs served as mock-transfected and positive controls for an ER-retained PT, respectively. In cells expressing PT α/β-Myc alone or PT α/β-Myc and Sar1
ER to Golgi Transport of PT α/β-Subunit Precursor Protein

In the present study, we have identified combinatorial trafficking signals in the PT α/β-subunit precursor protein controlling its ER to Golgi transport. Using the site-1 protease (PCSK9)-catalyzed cleavage of the α/β-subunit precursor protein and the acquisition of endoglycosidase H-resistant complex glycans on the mature β-subunit, the export from the ER and the arrival in the Golgi apparatus have been monitored. The data show that the ER export signal is composed by two distinct motifs, a dileucine signal (LL6) and a dibasic-based (1253RIR1255) motif localized in the cytosolic N-terminal domain and the extreme C terminus, respectively, of the PT α/β-subunit precursor protein. Additionally, expression analyses of mutant PT α/β-subunit precursor proteins containing missense mutations found in patients with MLIII revealed that distinct forms of the disease might be due to impaired transport to the Golgi apparatus and subsequent lack of phospho-transferase activation.

The alanine substitution of the 5LL6 signal in the N-terminal domain significantly reduced the transport of the PT α/β-subunit precursor protein to the Golgi, whereas mutations of single, double, or all three dibasic motifs in the C-terminal domain moderately affected the ER transport. The combinatorial mutations, however, of both dibasic export motifs in the C-terminal domain with the dileucine signal in the N-terminal domain of the PT α/β-subunit precursor protein strongly inhibited the ER export.
indicating the dominant role of the dileucine sorting motif. However, the substitution of the C-terminal \(^{1253}\text{RIR}^{1255}\) motif by dileucine residues either in combination with a dileucine motif in the N terminus \(^{5\text{LL}6/1253\text{LL}1254}\) or with a dialanine substitution \(^{5\text{AA}6/1253\text{LL}1254}\) prevented the ER exit of the \(\text{H9251/H9252}\)-subunit precursor protein and subsequently its proteolytic processing. The requirement of two distinct combinatorial sorting signals in defined positions of the N- and C-terminal domains of the \(\text{H9251/H9252}\)-subunit precursor protein for efficient ER export is without precedent. Furthermore, the data revealed that the two \(^{1236}\text{KRK}^{1238}\) and \(^{1242}\text{RRR}^{1244}\) motifs located close or seven residues away from the transmembrane domain, were less effective to promote ER exit than the distal \(^{1253}\text{RIR}^{1255}\) motif. This is in contrast to findings on members of the type II glycosyltransferase family demonstrating that the \((\text{RK})X(\text{RK})\) motif proximal to the transmembrane domain promotes the ER export most effectively (11). Prerequisite for dileucine/dibasic motif-dependent ER exit, however, is the type III membrane topology of the \(\text{H9251/H9252}\)-subunit precursor protein. Coexpression of the type II \(\text{H9251}\)-subunit with the type I \(\text{H9252}\)-subunit membrane protein failed to form a subunit complex competent to be sorted into ER-Golgi transport vesicles. It is likely that the dileucine/dibasic sorting motifs of the uncleaved \(\text{H9251/H9252}\)-subunit or the covalently linked tetrameric \(\text{H9251}_2/\text{H9252}_2\)-subunit precursor proteins have to be positioned in defined distances to each other and/or form a unique tertiary conformation which can be

**FIGURE 8.** Pathogenic mutation K4Q impairs the ER export of the PT \(\alpha/\beta\)-subunit precursor protein. **A**, COS-7 cells were transfected with EGFP, wild-type PT \(\alpha/\beta\)-Myc, PT \(\alpha/\beta\)-Myc-ER-Ret, and mutant PT \(\alpha/\beta\)-Myc K4Q and K1236M cDNA constructs. Twenty four hours after transfection, cell extracts (50 \(\mu\)g protein) were separated by SDS-PAGE and analyzed by Myc Western blotting (WB). Equal loading of the gel is shown by anti \(\alpha\)-tubulin immunostaining. The positions of the PT \(\alpha/\beta\)-subunit precursor protein and the cleaved PT \(\alpha/\beta\)-subunit are indicated. An asterisk indicates a nonspecific polypeptide. **B**, densitometric quantification reveals significant decreased ER export of mutant PT \(\alpha/\beta\)-Myc K4Q. The intensities of immunoreactive bands derived from immunoblots of three independent transfection experiments were quantified, and the fraction of cleaved PT \(\beta\)-subunit normalized to the total amount of PT are given as mean \(\pm\) S.D. in a bar diagram. The value determined in PT wild-type expressing cells was set as 1. *****, \(p < 0.0001\). **Black bar**, wild-type PT \(\alpha/\beta\)-Myc; **white bar**, PT \(\alpha/\beta\)-Myc-ER-Ret; **light gray bar**, PT \(\alpha/\beta\)-Myc K4Q; **dark gray bar**, PT \(\alpha/\beta\)-Myc K1236M. **C**, COS-7 cells were transfected with mutant PT \(\alpha/\beta\)-Myc K4Q and K1236M cDNA constructs and fixed 24 h after the start of transfection. Cells were probed with antibodies against Myc (green, panels a, d, g, and i), against the cis-Golgi marker protein GM130 (red, panels b and h), or the ER marker protein PDI (red, panels e and k). Nuclei were visualized with DAPI (blue) and merged images (yellow, panels c, i, f, and l) indicate co-localization. Scale bars, 10 \(\mu\)m.
recognition by the COPII machinery. Additionally, the present data suggest that the dileucine/dibasic sorting motifs of the α/β-subunit precursor protein interact with distinct interaction sites of COPII.

We showed that the ER export of the α/β-subunit precursor protein is inhibited in the presence of the dominant negative Sar1 mutant H79G, indicating its transport in COPII-coated vesicles. The COPII coat consists of the small GTPase Sar1, and the heteromeric protein complexes Sec23–Sec24 and Sec13–Sec31, which sequentially initiate the membrane curvature, cargo binding, and vesicle fission (27). Several studies have demonstrated that Sec24 binds LXXLE, diacidic DXE, and YNNSNP exit motifs in membrane proteins (28, 29). In addition, using the two membrane spanning protein Env41p as a model in prebudding in vitro experiments, it could be shown that the 549IL650 motif in the cytosolic C-terminal domain displayed Sec23–Sec24 binding activity, which was, however, not sufficient for ER export (30). Dibasic (RK)(X)(RK) ER export motifs present in cytoplasmic domains of type II membrane protein member of glycosyltransferases located proximal to the transmembrane border have been reported to interact directly with the COPII Sar1 component (11). At present, it is unknown which COPII component binds to the distal RIR motif of the PT α/β-subunit precursor protein and how LL and RIR determinants cooperatively interact with distinct coat proteins.

binorial signal structures for efficient ER export have also been observed in multisubunit transmembrane proteins Erv41p/Erv46p (30).

Two homozygous missense mutations, K4Q and K1236M, changing amino acids in the N- and C-terminal domains of the α/β-subunit precursor protein, respectively, have been identified in GNPTAB MLII/III patients (23, 24). Here, we show that the K4Q mutation reduced the proteolytic processing of the α/β-subunit precursor protein by 60–70%, which most likely impaired the 5IL6-dependent ER export rather than directly the proteolytic cleavage in the luminal domain between residues Lys-928–Asp-929. Because the efficiency of ER export strongly depends on the position of the dileucine signal and the context of the surrounding amino acids, the replacement of the positively charged lysine residue 4 by an uncharged glutamine might affect the interaction of the N-terminal domain with COPII components more dramatically than the 5IL6 alanine mutation. The reduced amounts of proteolytically activated α/β-subunit precursor protein K4Q is in agreement with the GlcNAc-1-phosphotransferase activity of 12% in fibroblasts of the patients and 20–40% of wild-type after transient expression in HEK293T cells (23). Because our data excluded 1236KKK1238 as active ER export signal, it is unlikely that the K1236M mutation affects the transport of the PT α/β-subunit precursor protein to the Golgi apparatus and subsequently allows its proteolytic cleavage. The intermediate phenotype of a patient who survived to the age of 14 years (24) suggests that the mutant GlcNAc-1-phosphotransferase exhibits residual activity. Further studies are needed to examine whether K1236M substitution impairs the assembly of α2β2 tetramers or α2β2γ2 hexamer complexes in the ER, which might be important for proper phosphorylation of all lysosomal hydrolases (7).

Taken together, we described a novel combinatorial dileucine and dibasic ER export motif located in the N- and C-terminal cytosolic domains of the type III GlcNAc-1-phosphotransferase α/β-subunit precursor membrane protein. Mutations affecting the accessibility of these export determinants with COPII components may impair the selection into transport vesicles, its subsequent proteolytic activation in the Golgi apparatus, and thus cause mucolipidosis type III.

Acknowledgments—We thank Dr. M. Schwake (University of Kiel, Germany) for the pFROG-mLIMP II-ER retention construct. The polyclonal anti-red fluorescent protein antibodies were kindly provided by Dr. H. J. Kreienkamp (University Medical Center Hamburg-Eppendorf, Germany), and the pCS2 mCherry vector was a kind gift of Dr. C. Weber (University Medical Center Hamburg-Eppendorf, Germany). We thank Helen Wegener for excellent technical assistance.

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