Proteolytic Processing of the γ-Subunit Is Associated with the Failure to Form GlcNAc-1-phosphotransferase Complexes and Mannose 6-Phosphate Residues on Lysosomal Enzymes in Human Macrophages*5

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GlcNAc-1-phosphotransferase is a Golgi-resident 540-kDa complex of three subunits, α2β2γ2, that catalyze the first step in the formation of the mannose 6-phosphate (M6P) recognition marker on lysosomal enzymes. Anti-M6P antibody analysis shows that human primary macrophages fail to generate M6P residues. Here we have explored the sorting and intracellular targeting of cathepsin D as a model, and the expression of the GlcNAc-1-phosphotransferase complex in macrophages. Newly synthesized cathepsin D is transported to lysosomes in an M6P-independent manner in association with membranes whereas the majority is secreted. Realtime PCR analysis revealed a 3–10-fold higher GlcNAc-1-phosphotransferase subunit mRNA levels in macrophages than in fibroblasts or HeLa cells. At the protein level, the γ-subunit but not the β-subunit was found to be proteolytically cleaved into three fragments which form irregular 97-kDa disulfide-linked oligomers in macrophages. Size exclusion chromatography showed that the γ-subunit fragments lost the capability to assemble with other GlcNAc-1-phosphotransferase subunits to higher molecular complexes. These findings demonstrate that proteolytic processing of the γ-subunit represents a novel mechanism to regulate GlcNAc-1-phosphotransferase activity and the subsequent sorting of lysosomal enzymes.

Soluble lysosomal enzymes are synthesized and co-translationally glycosylated in the endoplasmic reticulum (ER). Upon arrival in the Golgi apparatus they are specifically modified with mannose 6-phosphate (M6P) residues, which can be recognized by one of two specific M6P receptors with molecular masses of 300 kDa (MPR300) and 46 kDa (MPR46) in the trans-Golgi network. The MPR mediate the segregation of the lysosomal enzymes from the secretory pathway and delivery to endosomes and lysosomes (1). M6P residues are synthesized by the sequential action of two enzymes. First UDP-N-acetylglucosamine (UDP-GlcNAc): lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (termed GlcNAc-1-phosphotransferase) catalyzes the transfer of a GlcNAc-1-phosphate residue from UDP-GlcNAc to C6 positions of selected mannoses in high mannose-type oligosaccharides of the hydrolases (2). The second step involves the removal of the terminal GlcNAc by an N-acetylglucosamine-1-phosphodiester α-N-acetyl-glucosaminidase exposing the M6P recognition signal (2–4).

The bovine GlcNAc-1-phosphotransferase was found to be a hexameric 540-kDa enzyme complex consisting of three different subunits, α2β2γ2 (5). A single gene, GNPTAB, encodes the human α/β-subunit precursor protein that comprises 1,256 amino acid residues and spans the membrane twice (6, 7). It is believed that the α/β-subunit precursor is proteolytically cleaved into the individual subunits, which seems to be a prerequisite for the catalytic activity of the enzyme (8). The α- and β-subunit contain the catalytic activity and binding sites for lysosomal enzymes (9). The GNPTG gene encodes the γ-subunit of the GlcNAc-1-phosphotransferase, which represents a soluble glycoprotein of 305 amino acids capable of forming disulfide-linked dimers (10). The function of the γ-subunit is less clear, but it is thought to be involved in the regulation of activity and expression of the α/β-subunits (9–11). Mutations in the GNPTAB and GNPTG gene have been found in patients with mucolipidosis (ML) type II and III characterized by nonsorting of lysosomal enzymes lacking M6P residues, intracellular deficiency of acid hydrolases, and lysosomal storage of non-degraded material (reviewed in Refs. 12, 13).

In addition to the MPR-dependent transport of lysosomal enzymes, M6P-independent lysosomal target systems were...
described in various cell types and species such as rabbit macrophages, human hepatoma HepG2 cells, lymphoblasts of GlcNAc-1-phosphotransferase-deficient MLI1I patients, or in hepatocytes and thymocytes of Mpr-deficient mice (14–17). We have shown that the lysosomal enzymes cathepsin D and arylsulfatase A secreted from primary cultured human macrophages are barely equipped with M6P residues preventing an efficient uptake by acceptor cells (18). This observation led to the assumption that in these cells the function of the GlcNAc-1-phosphotransferase is impaired and lysosomal enzymes have to be transported to lysosomes in an M6P-independent manner.

In this study we used a novel single-chain antibody fragment against M6P residues (scFv M6P-1) to provide evidence that almost all proteins in human macrophages lack M6P residues. Using cathepsin D as a model we showed that in these cells both newly synthesized precursor and proteolytically processed forms are transported to lysosomes in an M6P-independent manner accompanied by a hypersecretion of the precursor polypeptide. Furthermore, the proteolytic cleavage of the γ-subunit in macrophages leads to the formation of unique oligomers that fail to associate with the GlcNAc-1-phosphotransferase subunits in higher molecular mass complexes. The data suggest that the limited proteolysis of γ-subunits represents a novel mechanism to regulate the GlcNAc-1-phosphotransferase activity and the subsequent sorting efficiency of lysosomal enzymes.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were obtained commercially as indicated: [35S]methionine, [125I]iodine, rainbow-colored protein molecular mass marker, molecular mass standards for size exclusion chromatography, and the bacterial expression vector pGEX-4T-1 from GE Healthcare, medium for cultivating Escherichia coli and octyl-β-D-glucopyranoside from Roth, M6P, protein A-agarose, saponin, bovine serum albumin (BSA), TRIReagent, glutathione-agarose, and protease inhibitor mixture from Sigma, Enhanced chemiluminescence from Pierce, PNGasE from Roche Diagnostics, Phusion® polymerase from Finnzymes, High Capacity cDNA Reverse Transcription Kit and TaqMan® Gene Expression Assays from Applied Biosystem. Maxima™ Probe qPCR Master Mix and PageRuler™ prestained protein ladder from Fermentas. Fetal calf serum from PAA, Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum and antibiotics at 37 °C and 5% CO2. COS7 cells were transiently transfected with the full-length cDNAs of the α/β-subunit precursor was generated by PCR with Phusion® polymerase using the full-length GNPTAB cDNA as template (7). The resulting PCR product was cloned into expression vector pcDNA3.1D/V5-His-TOPO according to the manufacturer’s instructions. The construct was sequenced on an ABI PRISM 377 DNA Sequencer (University Medical Center Service Laboratory, Hamburg-Eppendorf). The expression vector coding for the N-terminally Myc-tagged γ-subunit has been described elsewhere (11).

Cell Culture and Transfection—Primary macrophages were isolated from human buffy coats and cultivated for 14 days as described previously (18). Human fibroblasts, HeLa, MCF7, and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum and antibiotics at 37 °C and 5% CO2. COS7 cells were transiently transfected with the full-length cDNAs of the α/β-precuror or the γ-subunit of the GlcNAc-1-phosphotransferase using Lipofectamine™ 2000 according to the manufacturer’s instructions.

Size Exclusion Chromatography—Primary human macrophages or γ-subunit overexpressing COS7 cells grown on 60-mm plates were harvested and then resuspended in 100 μl of 10 mM PBS (pH 7.4) containing 30 mM octyl-β-D-glucopyranoside and protease inhibitor mixture. After 15-min incubation on ice, the samples were centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatant was diluted with an equal volume of ice-cold PBS (final 15 mM octyl-β-D-glucopyranoside). Cell extracts (50 μl) were loaded on a Superdex 200 PC 3.2/30-column equilibrated with 15 mM octyl-β-D-glucopyranoside/PBS, on a SMART HPLC system (Amerham Biosciences). Proteins were eluted with a flow rate of 40 μl/min, detected by UV absorption at 280 nm, collected in fractions of 100 μl and analyzed by Western blotting.

Metabolic Labeling and Permeabilization of Cells—Cells were grown on 35-mm dishes and labeled with [35S]methionine (50 μCi/ml) in the presence or absence of 10 mM NH4Cl for 16 h. Extracts of cells and medium were prepared and used for immunoprecipitation of cathepsin D as described previously (18). For differential permeabilization (14), cells were labeled with [35S]methionine (150 μCi/ml) for 30 min and chased for various times in serum-free medium containing 0.1% BSA. After removing the medium, the cells were chilled to 4 °C by three washes with ice-cold PBS, and incubated with 0.7

GlcNAc-1-phosphotransferase in Macrophages
GlcNAc-1-phosphotransferase in Macrophages

ml of PBS containing 0.2% saponin in the absence or presence of 10 mM M6P for 30 min on a rocking platform at 4 °C. Thereafter, the saponin-containing buffer was removed, and the cells were washed and solubilized as described (14). Cell extracts, saponin-containing buffer, and medium were used for immunoprecipitation of cathepsin D.

MPR-antibody Overlay—Cell extracts (50 μg protein) were separated by SDS-PAGE (8% acrylamide) and transferred onto nitrocellulose membranes. After blocking with 1% BSA in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20 (TBS-BSA), the membranes were incubated with labeled anti-MPR antibodies 21D3 or 2C2 (300,000 cpm/ml blocking buffer) for 4 h at room temperature followed by washing and autoradiography.

Western Blot Analysis—The expression of the γ-subunit of the GlcNAc-1-phosphotransferase in cell extracts or fractions after size exclusion chromatography was analyzed by Western blotting as described recently (11).

For the detection of the β-subunit of the GlcNAc-1-phosphotransferase in macrophage extracts (100 μg of protein) or fractions after size exclusion chromatography (100 μl), both preparations were separated by SDS-PAGE (10% acrylamide) and blotted onto nitrocellulose. After blocking with TBS-BSA, the membrane was incubated with anti-human β-subunit antisera (1:500) overnight followed by incubation with goat-anti-rabbit IgG-conjugated to horseradish peroxidase (1:10,000) in the same buffer. The immunoreactive bands were visualized by enhanced chemiluminescence.

The content of M6P-containing proteins in extracts of macrophages and fibroblasts (each 75 μg) and medium (300 μl) was analyzed by scFv M6P-1 Western blotting as described (21).

Other Methods—For double immunofluorescence microscopy primary macrophages were cultured for 14 days, trypsinized, and grown on glass coverslips for 24 h. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Nonspecific binding was blocked by 2 h of incubation in PBS containing 1% BSA (solution 1) at 4 °C. Cells were then incubated for 16 h with primary antibodies in solution 1 and 1 h with secondary antibodies. After five washes, the cells were embedded in Mowiol. Fluorescence was detected, and images were obtained using a Leica DMIRE2 digital scanning confocal microscope and ADOBE PHOTOSHOP software, respectively.

The analysis of GNPTAB and GNPTG mRNA expression relative to β-actin expression by real-time PCR were carried out as described recently (24). The relative mRNA expression in HeLa cells was set at 1. Data are the mean of three independent RNA preparations of 14-day cultured cells. For enzymatic deglycosylation of proteins, cell extracts were incubated in the presence or absence of 1 unit of PNGase F for 1 h at 37 °C as previously described (25).

RESULTS

Human Macrophages Lack M6P Residues—We have shown previously that cultured macrophages secrete weakly or not phosphorylated lysosomal hydrolases such as cathepsin D and arylsulfatase A, respectively (18). To examine the total amount of M6P-containing proteins, we analyzed cell extracts and conditioned media of human fibroblasts and macrophages by a novel anti-M6P Western blotting approach (21). At steady state, several M6P-containing polypeptides of different intensities were detected in the range between 26 and 140 kDa in extracts of human fibroblasts (Fig. 1A). By contrast, extracts of human macrophages cultured for 14 days contained only one prominent M6P-containing protein of 65 kDa and two weaker immunoreactive bands of 68 and 160 kDa. In medium of cultured fibroblasts several immunoreactive bands between 33 and 125 kDa were present, which represented most likely precursor proteins that failed to bind to MPRs in the Golgi apparatus (Fig. 1B). In the medium of primary macrophages a single prominent 90-kDa protein and a weaker 45-kDa immunoreactive polypeptide were observed.

M6P-independent Transport of Cathepsin D in Macrophages—To determine whether the intracellular transport of newly synthesized lysosomal enzymes is M6P-independent in human macrophages, the effect of the weak base NH₄Cl on the sorting and processing of newly synthesized cathepsin D was tested. The lysosomotropic amine dissipates acid pH-dependent processes including the dissociation of MPR-lysosomal enzyme complexes in prelysosomal compartments (26). This treatment results in a rapid depletion of unoccupied MPR in the Golgi apparatus and hypersecretion of lysosomal enzymes into the medium (23, 27).

Macrophages and for comparison MCF7 cells were metabolically labeled with [³⁵S]methionine for 16 h in the presence or absence of 10 mM NH₄Cl followed by immunoprecipitation of cathepsin D. From extracts of MCF7 cells three
forms of cathepsin D could be immunoprecipitated (Fig. 2, lane 5). The 53-kDa precursor form is proteolytically processed in the prelysosomal/endosomal compartment to the 47-kDa intermediate form (28, 29). Both forms are delivered to lysosomes and cleaved to the mature 31- and 14-kDa forms. About 39% of cathepsin D precursor forms are secreted into the medium (Fig. 2, lane 6). Exposure of MCF7 cells to NH4Cl increased the amount of missorted cathepsin D to 73% (Fig. 2, lane 8). The intracellular transport to lysosomes and the proteolytic processing are interrupted as shown by the absence of intermediate and mature cathepsin D forms in the cell extracts (Fig. 2, lane 7). By contrast, in macrophages four forms of cathepsin D were detected. In addition to the precursor, intermediate and mature forms of 53, 47, and 31 kDa, respectively, the formation of a second 35/36-kDa intermediate form was observed (Fig. 2, lane 1), which disappeared after longer chase periods. About 46% of the total newly synthesized cathepsin D was recovered from the medium as highly glycosylated precursor resulting in a slight reduction of the electrophoretic mobility (Fig. 2, lane 2). In the presence of NH4Cl the formation of the mature and the 35/36-kDa intermediate form of cathepsin D was strongly reduced or prevented, respectively, in macrophages (Fig. 2, lane 3) whereas neither the processing to the 47-kDa intermediate form nor the percentage of secreted cathepsin D (Fig. 2, lane 4) were changed. These data indicate that in macrophages the NH4Cl-induced increase in pH neither affects the sorting of cathepsin D in the Golgi apparatus nor the processing to the intermediate forms occurring in endosomes. The lack of NH4Cl-induced missorting of newly synthesized cathepsin D is characteristic of an MPR-independent transport (30, 31). The proteolytic processing of cathepsin D in lysosomes, however, depends on low pH and is inhibited by NH4Cl. The usage of an alternative transport system is not induced by low expression of the MPRs because overlay analysis of cell extracts with 125I-labeled antibodies against MPR revealed that both MPR300 and MPR46 are expressed in macrophages at a similar level as in control cells (Fig. 3).

M6P-independent Association of Cellular Cathepsin D Forms with Membranes—To analyze the M6P-independent transport steps in more detail the kinetics of sorting and processing of newly synthesized cathepsin D has been determined in macrophages by pulse-chase experiments. Within 1 h after synthesis the first traces of 35S-labeled cathepsin D precursor forms could be immunoprecipitated from the medium (Fig. 4, lanes 5 and 6). This fraction increased continuously during the chase period of 6 h (Fig. 4, lanes 5–8). The first intracellular processed intermediate cathepsin D form appeared after 3 h of chase (Fig. 4, lane 3). Six hours after synthesis about 35, 37, and 9% of intracellular cathepsin D were immunoprecipitated as precursor, intermediate, and mature form, respectively (Fig. 4, lane 4).

M6P-independent transport of cathepsin D has been demonstrated in HepG2 cells by differential permeabilization of membranes (14). The M6P-independent membrane association of cathepsin D during its transport along the biosynthetic pathway in macrophages is shown in Fig. 5. Macrophages were labeled for 30 min with [35S]methionine and chased for 30–240 min. The cells were then either directly used for immunoprecipitation of cathepsin D (Fig. 5, lanes 1 and 6) or partially permeabilized with saponin allowing the release of cytosolic and soluble lumenal proteins into the supernatant. Upon treatment of macrophages with saponin 30 min after synthesis about 70% of newly synthesized cellular cathepsin D was released into the supernatant (S) as precursor form (Fig. 5, lanes 2 and 3). There was no observable increase of this fraction in the presence of 10 mM M6P (Fig. 5, lane 5), confirming that M6P residues do not contribute to the binding of cathepsin D to membranes of macrophages. After 240 min of chase, the 47- and 31-kDa intermediate and mature cathepsin D forms were found to be membrane-associated (Fig. 5, lane 7) in an M6P-independent manner (Fig. 5, lane 9). About half of the newly synthesized cathepsin D precursor recovered from cells can be released from intracellular membranes upon saponin permeabilization (Fig. 5, lanes 8 and 10). The data show that
GlcNAc-1-phosphotransferase in Macrophages

| chase (min) | 30 | 90 | 180 | 360 | 30 | 90 | 180 | 360 |
|------------|----|----|-----|-----|----|----|-----|-----|
| 46 kDa     |    |    |     |     | p  |    |     |     |
| 30 kDa     |    |    |     |     | m  |    |     |     |

**FIGURE 4.** Time-dependent intracellular sorting and secretion of newly synthesized lysosomal enzymes in human macrophages. Macrophages cultured for 14 days were labeled for 30 min with $[^{35}S]$methionine (150 μCi/ml) and chased in radioactive-free medium for different time periods shown as indicated. Cathepsin D was immunoprecipitated from cell extracts and medium, and samples were analyzed by SDS-PAGE and fluorography. The position of molecular mass marker proteins in kDa, precursor (p), intermediate (i), and mature (m) forms of cathepsin D are indicated.

| chase (min) | 30 | 240 |
|------------|----|-----|
| saponin    | +  | +   |
| M6P        |   |     |

**FIGURE 5.** M6P-independent association of cathepsin D forms with macrophage membranes. Macrophages cultured for 14 days were labeled for 30 min with $[^{35}S]$methionine (150 μCi/ml) and chased for 30 or 240 min. The cells were chilled to 4 °C and permeabilized with PBS containing 2 mg/ml saponin with (+) or without (−) 10 mM M6P for 30 min. Cathepsin D was immunoprecipitated from cell extracts (C) and PBS-saponin supernatants (S). Samples were analyzed by SDS-PAGE and fluorography. The positions of molecular mass marker proteins in kDa, precursor (p), intermediate (i), and mature (m) forms of cathepsin D are indicated.

TABLE 1
Relative expression of mRNA of GlcNAc-1-phosphotransferase subunits in human macrophages

| Cells     | GNPTAB | GNPTG |
|-----------|--------|-------|
| HeLa      | 1.0 ± 0.5 | 1.0 ± 0.2 |
| Fibroblasts | 3.0 ± 0.6 | 2.6 ± 0.7 |
| Macrophages | 7.4 ± 1.1 | 9.8 ± 1.8 |

newly synthesized cellular cathepsin D is transported to lysosomes in association with membranes in an M6P-independent manner. Furthermore, membrane-bound cathepsin D can be proteolytically processed into intermediate and mature forms.

**Expression of the GlcNAc-1-phosphotransferase in Macrophages**—To examine whether the lack of M6P residues on lysosomal enzymes in macrophages is caused by an altered expression of the GlcNAc-1-phosphotransferase, first the relative mRNA levels of GNPTAB and GNPTG encoding the α/β-subunit precursor and the γ-subunit, respectively, were determined by real-time PCR. For comparison the expression of both genes was analyzed in HeLa cells and human fibroblasts. The relative mRNA expression in macrophages of GNPTAB was 7.4- and 2.5-fold and of GNPTG 9.8- and 3.8-fold higher than in HeLa cells and fibroblasts, respectively (Table 1).

Second, double immunofluorescence microscopy demonstrated the presence of immunoreactive γ-subunit polypeptides in the Golgi apparatus of macrophages by complete colocalization with the cis-Golgi marker GM130 (Fig. 6A). No colocalization was observed with marker proteins of the ER (PDI, not shown) or lysosomes (LAMP1, Fig. 6B). Confocal stacks of series of single images for each staining are shown in the supplemental Fig. S1.

Third, cell extracts of macrophages were analyzed for the expression of GlcNAc-1-phosphotransferase β- and γ-subunits by Western blotting. The anti-human β-subunit antibody recognizes a 190-kDa α/β-precursor protein as well as the proteolytically cleaved mature 45-kDa β-subunit in overexpressing COS7 cells that were not detectable in non-transfected cells (Fig. 7A). Upon treatment with PNGase F, the 135-kDa and 38-kDa deglycosylated α/β-subunit pre-cursor and the mature β-subunit forms, respectively, were observed. In macrophages only the endogenous mature β-subunit polypeptide is detectable at steady state exhibiting the same extent of glycosylation as the overexpressed β-subunit (Fig. 7A). Endogenous β-subunits were not detectable in human fibroblasts or HeLa cells most likely due to their low expression. The γ-subunit of the GlcNAc-1-phosphotransferase forms disulfide-linked dimers, which can be transformed into monomers under reducing conditions (10). When cell extracts of macrophages were analyzed for the expression of dimeric γ-subunits, a 97-kDa immunoreactive polypeptide was detectable, which showed an increased electrophoretic mobility upon PNGase F treatment (Fig. 7B). Overexpression of the γ-subunit cDNA in COS7 cells, instead, gave rise to a 72-kDa γ-subunit dimer which was absent in non-transfected cells (Fig. 7B). Upon treatment with PNGase F the molecular mass of the glycosylated 72-kDa dimer shifted to 62 kDa as expected from the length of the cDNA. Under reducing conditions a 36-kDa glycosylated and 31-kDa deglycosylated γ-subunit was observed in overexpressing COS7 cells. In macro-
phages immunoreactive γ-subunit polypeptides of 23–28 kDa were observed, which were converted to the 23-kDa size upon treatment with PNGaseF (Fig. 7C). Higher resolution Tricine SDS-PAGE of macrophage extracts revealed the formation of three immunoreactive γ-subunit fragments of 28, 24, and 23 kDa (Fig. 7D). The data indicate that in human macrophages the γ-subunit of the GlcNAc-1-phosphotransferase is proteolytically cleaved and forms irregular sized oligomeric complexes.

**Proteolytic γ-Subunit Fragments Fail to Form High Molecular Mass GlcNAc-1-phosphotransferase Complexes**—It has been reported that the three types of subunits of bovine GlcNAc-1-phosphotransferase form a 540-kDa hexameric αβγγγγ complex (5). To examine the biological significance of proteolytic cleavage of the γ-subunit in macrophages, their capability to form high molecular mass complexes was analyzed by size exclusion chromatography. For comparison the elution profiles of octyl-β-D-glucopyranoside extracts of COS7 cells overexpressing the human γ-subunit was analyzed. Fig. 8B shows that all immunoreactive γ-subunits were exclusively recovered from fraction 2. Comparison with the separation of protein standards (Fig. 8A) indicates that fraction 2 displays molecular masses of ~660 kDa. These data indicate that the overexpressed human γ-subunit forms higher molecular mass complexes with endogenous proteins in COS7 cells. When extracts of human macrophages were fractionated by size exclusion chromatography the majority of the immunoreactive 28-kDa γ-subunit eluted in fractions 9 and 10. The 23-kDa γ-subunit fragment was found in fractions displaying molecular masses ≥66 kDa (fractions 7 and 8, Fig. 8C). No immunoreactive γ-subunit polypeptides were observed in fraction 2 co-eluting with high molecular mass protein complexes. The elution profile and the distribution of the endogenous 45-kDa β-subunit of GlcNAc-1-phosphotransferase of macrophages are shown in Fig. 8D demonstrating that the β-subunit is detectable in fraction 2 (660 kDa). These data are consistent with the conclusion that in human macrophages the γ-subunit fragments fail to form higher molecular mass complexes with β- (and α-) subunits of the GlcNAc-1-phosphotransferase.
In this study cathepsin D was used as a model to analyze the trafficking of lysosomal acid hydrolases in cultured human macrophages. In these cells the stoichiometry of M6P residues on newly synthesized cathepsin D is low (18). Western blot analysis using the novel single chain anti M6P antibody fragment scFv M6P-1 provided evidence that almost all cellular and secreted proteins of macrophages lack the M6P recognition marker. In addition the sorting of newly synthesized cathepsin D was not sensitive to treatment of macrophages with the weak base NH₄Cl, characteristic of an M6P receptor-independent transport (23, 30). Furthermore, differential permeabilization of pulse-labeled macrophages with saponin showed that cathepsin D was synthesized as a soluble protein and acquired its membrane-association 30 min after synthesis. This membrane association was, as expected, insensitive to M6P. The kinetic data indicate that the membrane association of cathepsin D precursor forms occur in the Golgi apparatus and appear to be maintained also during proteolytic processing to 47- and 31-kDa forms in endosomes and lysosomes, respectively. These data indicate that primary cultured macrophages are defective in the synthesis of M6P residues on lysosomal proteins rather than exhibiting an increased M6P phosphatase activity proposed to be an endosomal/lysosomal event (32, 33).

The present results on lysosomal trafficking of cathepsin D in human macrophages differ from studies in HepG2 cells where cathepsin D and the lysosomal sphingolipid-activating protein (SAP) are transported both in MPR-dependent and independent manner (14). Furthermore, it has been shown that the membrane-association of cathepsin D in HepG2 cells prevents the proteolytic processing. The receptor proteins mediating the M6P-independent membrane association in macrophages and HepG2 hepatoma cells are still unknown. A candidate for an alternative receptor protein for SAP is sortilin also named neutrotensin receptor 3 (34). In addition, it has been demonstrated that secreted SAP can be reinternalized in several cell lines such as mouse and human fibroblasts, and murine neuroblastoma derived C-46 cells by the multifunctional low density lipoprotein receptor-related protein (LRP), and in murine macrophages by mannose receptors (35). For another soluble lysosomal hydrolase, glucocerebrosidase that does not acquire M6P residues (36), the lysosomal integral membrane protein LIMP-2 was identified as its lysosomal targeting receptor recently (37).

Defects in the formation of M6P residues are found in patients with mucolipidosis (ML) II and MLIII (12, 13) caused by mutant α-, β-, or γ-subunits comprising the GlcNAc-1-phosphotransferase complex. Sequencing of both GNPTAB and GNPTG genes in cultured macrophages failed to detect any mutations. Furthermore, quantitative real-time PCR showed that the relative mRNA level of both GNPTAB and GNPTG were elevated to a similar extent compared with those in human fibroblasts or Hela cells (Table 1), indicating the stability of mRNAs. In contrast, a characteristic finding in fibroblasts of MLIII patients exhibiting mutations in GNPTG gene, is the compensatory increase in GNPTAB mRNA expression (11, 24), which was not observed in macrophages. These data support the idea that neither genomic nor transcriptional alterations in GNPTAB/GNPTG are responsible for the loss in GlcNAc-1-phosphotransferase activity in macrophages.

The unexpected observation made in this study was the proteolytic processing of GlcNAc-1-phosphotransferase γ-subunits into three fragments, which were able to form irregular cysteine-linked 97-kDa oligomers. These γ-subunit fragments lost their capability to assemble with other GlcNAc-1-phosphotransferase subunits to higher mass complexes as shown by size exclusion chromatography. At present, however, it is not

![Figure 8: Subunit assembly of the GlcNAc-1-phosphotransferase in COS7 cells and human macrophages.](image-url)
clear whether the failure of assembly is responsible for the loss in GlcNAc-1-phosphotransferase activity. In the absence of the γ-subunit in brain tissue of Gnpptg-targeted mice the phosphorylation of lysosomal enzymes is partially reduced and differs among various acid hydrolases (9, 38). In fibroblasts of patients, however, the absence of the γ-subunit caused by mutations in GNPTG that result e.g. in premature translational termination and mRNA decay (p.V176GfsX18, p.G204YfsX17, or p.Q203HfsX3), lead to the loss of the M6P recognition marker on lysosomal enzymes (24, 39). In addition mutations in GNPTAB encoding the α/β-subunit precursor result in a secondary degradation of the γ-subunit (40, 41) and the associated loss of the M6P marker. Whereas proteolytic modifications of the β-subunit can be excluded in macrophages, no data are available on the α-subunit polypeptide because specific antibodies are lacking. Thus, the data of this study provide evidence that the formation of proteolytic fragments of the γ-subunit represents a novel mechanism to regulate GlcNAc-1-phosphotransferase activity. The identification of the protease localized at the lumenal site in or proximal of the cis-Golgi, and the physiological conditions initiating the proteolytic cleavage of the γ-subunit in macrophages remain to be investigated. Surprisingly, the γ-subunit fragments that lost their capability to assemble with β- (and most likely also α-) subunits, are still localized in the Golgi apparatus of macrophages rather than being secreted into the culture medium. This suggests that the γ-subunit fragments or their irregular oligomers may interact with other so far unidentified proteins mediating their retention in the Golgi apparatus.

The secretion of lysosomal enzymes from hematopoietic monocytes/macrophages and the re-uptake by surrounding enzyme-defective cells is considered the rationale for allogeneic bone marrow transplantation (BMT) as therapeutic approach for lysosomal storage disorders with central nervous system manifestation. Bone marrow-derived macrophages can cross the blood-brain barrier and differentiate into perivasculary macrophages and microglia (42, 43). In β-hexosaminidase-deficient mice, however, BMT did neither increase the β-hexosaminidase level in neurons nor affect the glycolipid storage in the brain (44, 45). The minor improvement of neurological symptoms and the increased life span in these mice after BMT are attributed to the suppression of microglia activation and neuronal death, rather to enzyme secretion/recapture mechanisms to correct the enzyme deficiency of brain cells (46). The lack of M6P residues on lysosomal enzymes secreted by macrophages/microglial cells may explain the failure of affected brain cells to recapture these enzymes efficiently.

Taken together, the present data provide evidence of a novel mechanism to regulate GlcNAc-1-phosphotransferase activity by limited proteolysis of its γ-subunit that subsequently may modulate the efficiency of protein transport to lysosomes. The γ-subunit fragments have lost their capability to form higher molecular mass complexes with other subunits of the GlcNAc-1-phosphotransferase and prevent the formation of M6P residues. The regulation of M6P synthesis at least in macrophages and the use of alternative cargo receptors may allow variations in the sorting and targeting of individual lysosomal hydrolases, which is important to adapt to inflammatory signals, changes in metabolism, or host defense.

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