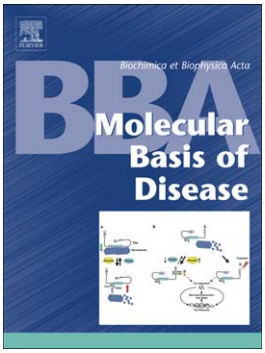

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Compensatory expression of human *N*-Acetylglucosaminyl-1-phosphotransferase subunits in mucolipidosis type III gamma

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Abstract

The *N*-Acetylglucosaminyl-1-phosphotransferase plays a key role in the generation of mannose 6-phosphate (M6P) recognition markers essential for efficient transport of lysosomal hydrolases to lysosomes. The phosphotransferase is composed of six subunits (α_2, β_2, γ_2). The α- and β-subunits are catalytically active and encoded by a single gene, *GNPTAB*, whereas the γ-subunit encoded by *GNPTG* is proposed to recognize conformational structures common to lysosomal enzymes. Defects in *GNPTG* causes mucolipidosis type III gamma, which is characterized by missorting and cellular loss of lysosomal enzymes leading to lysosomal accumulation of storage material. Using plasmon resonance spectrometry, we showed that recombinant γ-subunit failed to bind the lysosomal enzyme arylsulfatase A. Additionally, the overexpression of the γ-subunit in COS7 cells did not result in hypersecretion of newly synthesized lysosomal enzymes expected for competition for binding sites of the endogenous phosphotransferase complex. Analysis of fibroblasts exhibiting a novel mutation in *GNPTG* (c.619insT, p.K207IfsX7) revealed that the expression of *GNPTAB* was increased whereas in γ-subunit overexpressing cells the *GNPTAB* mRNA was reduced. The data suggest that the γ-subunit is important for the balance of phosphotransferase subunits rather for general binding of lysosomal enzymes.

Introduction

Newly synthesized soluble lysosomal enzymes are specifically modified in the Golgi apparatus with mannose 6-phosphate (M6P) residues. In a first step, the *N*-Acetylglucosaminyl-1-phosphotransferase (termed GlcNAc-1-phosphotransferase, EC 2.7.8.15) transfers *N*-Acetylglucosamine-1-phosphate to C6-position of mannose residues on high-mannose type oligosaccharides using UDP-GlcNAc as substrate [1, 2]. The second step involves the removal of the terminal GlcNAc to expose the M6P residues by a GlcNAc-1-phosphodiester *N*-Acetylglucosaminidase (NAGPA) residing in the *trans*-Golgi network [3]. M6P residues function as recognition marker required for the M6P receptor mediated transport to lysosomes. Along the biosynthetic pathway several lysosomal enzymes are proteolytically modified leading to mature, active enzymes. Varying amounts of newly synthesized lysosomal enzymes escape the binding to M6P receptors in the Golgi and are secreted [4].

The GlcNAc-1-phosphotransferase forms a 540 kDa hexameric complex composed of three subunits ($\alpha_2\beta_2\gamma_2$) [5]. A single gene, *GNPTAB* (MIM#607840), encodes the α/β-subunit precursor that spans the membrane twice [6, 7]. It is believed that the α/β-precursor is proteolytically processed into the individual subunits which seems a prerequisite for the catalytic activity of the enzyme. The *GNPTG* gene (MIM#607838) encodes the γ-subunit (GNPTG) which represents a soluble glycoprotein of 305 amino acids capable to form disulfide-linked dimers [8]. While it is thought that the α- and β-subunits of the GlcNAc-1-phosphotransferase comprise the binding sites of the UDP-GlcNAc substrate, and the catalytic activity [6, 9], little is known about the role of the γ-subunits for the GlcNAc-1-phosphotransferase complex.

Defective GlcNAc-1-phosphotransferase causes two distinct human lysosomal storage diseases, mucolipidosis types II and III (MLII, MIM#252500 and MLIII, MIM#252600, respectively). The clinically severe MLII (also called I-cell disease) is characterized by a

total loss of GlcNAc-1-phosphotransferase activity and associated with mutations in the *GNPTAB* gene [6, 10-13]. Mutations in the *GNPTG* gene are found in patients with MLIII [8, 14, 15]. However, several patients clinically diagnosed with MLIII were found to be homozygous or compound heterozygous for mutations in the *GNPTAB* gene [10, 11, 16] leading to a new classification of mucolipidosis type II and III as MLII alpha/beta and MLIII alpha/beta, or MLIII gamma considering both biochemical and genetic defects [17]. In both MLII and MLIII patients newly synthesized lysosomal enzymes failed to be sorted correctly to lysosomes due to the absence or weak equipment with M6P-residues resulting in lysosomal dysfunction and accumulation of non-degraded material [1].

In this study, we identified a novel mutation in *GNPTG* occurring in homozygosity in a boy with MLIII gamma resulting in a truncated but stable γ-subunit protein. *In vitro* and *in vivo* experiments as well as quantitative mRNA expression analysis revealed that the γ-subunit appears to be involved in the regulation of the GlcNAc-1-phosphotransferase activity rather than the general binding of lysosomal enzymes.

Materials and methods

Reagents and antibodies

Recombinant GNPTG was purified as a secretory 6His-fusion protein as described previously [6]. Recombinant arylsulfatase A (ASA) was produced and purified as described recently [18].

The reagents were obtained as indicated: Dulbecco's minimal essential medium (DMEM), Lipofectamine 2000™ from Invitrogen (Karlsruhe, Germany); fetal calf serum (FCS) from PAA Laboratories (Pasching, Austria); Chang medium®-In Situ (Irvine Scientific, Santa Ana, CA); [^{35}S]-methionine from Perkin Elmer Life Sciences (Boston, MA); protease inhibitor cocktail and TRI®-Reagent from Sigma-Aldrich (Deisenhofen, Germany); enhanced chemiluminescence (ECL) reagent from Pierce (Rockford, IL); Protein Assay from Bio-Rad (Hercules, CA); Rainbow™ protein marker from Amersham (Buckinghamshire, UK); Proofstart DNA Polymerase, QIAquick Gel Extraction from Qiagen (Hilden, Germany); High Capacity cDNA Reverse Transcription Kit and TaqMan® Gene Expression Assays from Applied Biosystem (Foster City, CA); Maxima™ Probe qPCR Master Mix, FastRuler™ DNA ladders and restriction enzymes from Fermentas (St.Leon-Rot, Germany); Primers were synthesized by MWG Biotech (Munich, Germany). Polyclonal antibodies against human GNPTG and human cathepsin D were described previously [15, 19]. The monoclonal antibody against β-tubulin was purchased from the NICHD Developmental Studies Hybridoma Bank (University of Iowa, IA). Secondary horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Dianova (Hamburg, Germany).

Cell culture and transfections

COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Cells were transfected with myc-*GNPTG* cDNA

using Lipofectamine 2000™ according to manufacturer´s instructions and selection was performed with neomycin (0.8 mg/ml). Fibroblasts of four control and three MLIII gamma patients were maintained in Chang medium®-In Situ supplemented with antibiotics. MLIII patient 1 and 2 were homozygous for c.500insC (p.V168Rfs29) and c.347_349delACA (p.N116del) in *GNPTG*, respectively and were described previously [14, 15].

Patient 3 was referred for biochemical analysis at the age of 2 ½ years on the assumption of mucopolysaccharidosis because of bone changes (dysostosis) by Dr. Niethammer, Esslingen (Germany). Due to the defiency of activities of several lysosomal enzymes in cultured fibroblasts of the patient and grossly elevated lysosomal activities in the serum (Suppl. table 1) the diagnosis of mucolipidosis type III was confirmed. The boy is the second child of non-consanguineous German parents. Today he is 13 years old with normal intelligence.

Sequencing and mutation analysis

The 11 exons of the *GNPTG* gene were amplified by PCR as described [14]. After separation of the PCR reaction mix by 2% agarose gel electrophoresis the products were excised and purified by QIAquick Gel Extraction Kit. Sequence reactions were performed with BigDye Terminator Reaction Kit (Applied Biosystems) and samples were sequenced on an ABI PRISM 377 DNA Sequencer (in the University Hospital Service Center, Hamburg). The DNA sequences were compared with published *GNPTG* sequences (GenBank: BC014592). Nucleotide numbering was referred to the ATG start codon, using the A as +1.

Construction of myc-tagged GNPTG

The expression constructs for myc-GNPTG were generated by PCR with Proofstart DNA Polymerase using the human *GNPTG* cDNA as template and the primers: myc-GNPTG-

For: 5´-CACCGAACAAAAACTCATCTCAGAAGAGGATCTGTGAATGGCGGCGGG GCTGGCGCGG-3´ and GNPTG-Rev: 5´-CAAACTCCCACGCAGTCCTGGGTC-3´. The resulting PCR product was cloned into the expression vector pcDNA3.1D/V5-His-TOPO® (Invitrogen) according to the manufacturer´s instructions. All constructs were sequenced as described above.

Surface plasmon resonance spectrometry

The BIAcore sensor chip (Amersham Biosciences) was activated by N-hydroxy-succinimide and N-ethyl-N`-(dimethyl amino propyl)-carbodiimide. The coupling of the ligands was carried out in 10 mM sodium acetate buffer (pH 5.0) with a ligand concentration of 50 µg/ml and a flow rate of 5 µl/min up to approx. 500 measured RUs on the sensor chip. Residual binding sites were blocked with 1 M ethanol amine hydrochloride (pH 8.5). Both ASA and recombinant GNPTG were used as analytes and as ligands, respectively. The analytes were solved in degassed and filtered 0.01 M Hepes/OH (pH 7.4), 0.15 M sodium chloride, 0.005% polysorbate 20 (v/v). The measurements were carried out with a flow rate of 10 µl/min and an injecting volume of 75 µl using 250-1000 nM ASA or 400 and 800 nM GNPTG in a BIAcore 3000 and calculated with the BIAEvaluation® software.

Metabolic labelling of cells and immunoprecipitation

Metabolic labelling of non-transfected and GNPTG overexpressing COS7 cells with [^{35}S]-methionine followed by immunoprecipitation of cathepsin D, SDS-PAGE and fluorography was carried out as described previously [19]. AIDA software (Raytest, Straubenhardt, Germany) was used for densitometric evaluation of immunoreactive band intensities.

Quantitative real-time PCR

Total RNA was isolated from fibroblasts or COS7 cells using the TRI®-Reagent. For cDNA synthesis 1 µg of total RNA and the High Capacity cDNA Reverse Transcription Kit were used according to the manufacturer's instructions. For real-time PCR, TaqMan® Gene Expression Assays including pre-designed probes and primer set for human *GNPTG* (Hs00261332_m1), *GNPTAB* (Hs00225647_m1) and *NAGPA* (Hs00212354_m1) were used. *β-Actin* was chosen as house keeping gene (*ACTB:* Hs9999903_m1*).* Real-time PCR reactions were performed as previously described [20]. The relative expression of analyzed mRNAs was normalized to the level of *ACTB* mRNA in the same cDNA sample using the comparative C_T method ($2^{-\Delta\Delta CT}$) [21].

:

Other methods

Postnuclear supernatants (PNS) were prepared in 10 mM Tris/HCl (pH 7.5) containing 250 mM sucrose and protease inhibitor cocktail. The cells were homogenized by 25 strokes with a 17-gauge needle on ice. After centrifugation at 1,000 *g* PNS were used for measurement of protein content by the Protein Assay reagent. Aliquots of PNS (25–75 µg protein) were separated by SDS-PAGE and blotted onto nitrocellulose. After blocking with 5% (w/v) non-fat dry milk in phosphate-buffered saline containing 0.1% (v/v) Tween-20, the membrane was incubated either with anti-human GNPTG antibody (1 : 250) or anti β-tubulin (1 : 1,000) overnight followed by incubation with respective secondary horseradish peroxidase-conjugated IgG (1 : 10,000) in the same buffer. The immunoreactive bands were visualized by enhanced chemiluminescence. The activities of the lysosomal enzymes in cell extracts and media were determined as described previously [15] and lysosomal enzymes activities in the patient´s serum as detailed elsewhere [16] .

Results

Identification of a novel mutation in the *GNPTG* gene in an MLIII gamma patient

The clinical and biochemical findings of patient 3 were compatible with the diagnosis of mucolipidosis type III. The activities of several lysosomal enzymes were increased in the serum (Suppl. Table 1). In cultured fibroblasts residual lysosomal enzyme activities varied between 8% (β-hexosaminidase, α-mannosidase) and 47% (galactocerebrosidase) of control cells. To examine whether the γ-subunit of the GlcNAc-1-phosphotransferase (GNPTG) was defective, the gene was sequenced in genomic DNA of the patient. A novel homozygous nucleotide insertion in exon 9 (c.619insT) was identified leading to a frameshift and to a premature stop codon after 7 new amino acids followed by the substituted Lys-207 residue (p.K207IfsX7). Western Blot analysis showed that in cell extracts of patient 3 fibroblasts a 24 kDa immunoreactive polypeptide was detectable whereas control cells expressed an immunoreactive polypeptide with the predicted molecular mass of 34 kDa of the GNPTG monomer (Fig. 1A). In contrast to the abundant truncated GNPTG protein, the relative *GNPTG* mRNA expression was 3-fold reduced compared with control cells (Fig. 1B). The mRNA expression of *GNPTG* was also 2- and 5-fold decreased in two other cell lines from MLIII gamma patients 1 and 2 with mutant GNPTG (Fig. 1B).

Compensatory gene expression in GNPTG-defective cells

To examine whether defects in the *GNPTG* gene affect the expression of other components involved in M6P formation, the mRNA levels of *GNPTAB* and *NAGPA* were determined in fibroblasts of the MLIII gamma patients. In MLIII gamma cell lines of patient 1, 2 and 3 the relative expression of both *GNPTAB* and *NAGPA* were 1.5- to 2.5-fold increased in comparison with the expression of four control cells (Fig. 1B).

GNPTG of the GlcNAc-1-phosphotransferase failed to bind arylsufatase A and cathepsin D

It is believed that the α/β-subunits (GNPTAB) of GlcNAc-1-phosphotransferase exhibit enzymatic activity whereas the γ-subunit (GNPTG) is responsible for binding of lysosomal enzymes [1]. To examine whether GNPTG is capable to bind lysosomal enzymes, surface plasmon resonance spectroscopy was used. Monomeric and dimeric forms of GNPTG were immobilized on the sensor chip**,** and recombinant arylsulfatase A (ASA) was passed over the surface (Fig 2A). After completion of analyte (ASA) injection, buffer change leads to detachment of the analyte indicating that ASA does not bind to immobilized GNPTG. Similarly, GNPTG failed to bind to immobilized ASA on the sensor surface, independently of analyte concentrations (Fig. 2B).

In a second approach the capability of GNPTG to function in the recognition of lysosomal enzymes was studied *in vivo*. GNPTG was stably overexpressed in COS7 cells to examine whether it can compete with the endogenous GlcNAc-1-phosphotransferase complex for binding of newly synthesized lysosomal enzymes. Subsequently the formation of M6P residues should be reduced which should lead to missorting of newly synthesized lysosomal enzymes into the medium and decreased enzymatic activities of intracellular lysosomal hydrolases. Western blot analysis showed the expression of a 36 kDa immunoreactive polypeptide, which was weakly detectable in non-transfected COS7 cells (Fig. 3A). Real-time PCR analyses revealed that the *GNPTG* mRNA levels in the transfected cells were approximately 28-fold higher than in non-transfected cells (Fig. 3B). The expression of *GNPTAB* was significantly reduced in *GNPTG* overexpressing COS7 cells whereas the level of *NAGPA* mRNA was not affected (Fig. 3B).

Next, the transport and sorting of the newly synthesized lysosomal enzyme cathepsin D was examined in GNPTG overexpressing COS7 cells. Cells were labelled for 1 hour with [^{35}S]-methionine and either harvested or chased for further 4 hours in non-radioactive

media followed by immunoprecipitation of cathepsin D from cell extracts and media. After pulse-labelling, the majority of cathepsin D could be precipitated as a 53 kDa precursor protein, and only small amounts of a 31 kDa immunoreactive polypeptide were detectable (Fig. 4). During the chase period both in non-transfected and in GNPTG overexpressing cells the cathepsin D precursor polypeptide was completely processed to the mature form. Under these conditions 17 and 23% of the newly synthesized cathepsin D precursor were secreted into the media (Fig. 4). Furthermore, in comparison with non-transfected cells we found no differences in activities of the lysosomal enzymes β-hexosaminidase, β-glucuronidase, and ASA in cells and media of GNPTG overexpressing COS7 cells (Suppl. Table 2). The data suggest that GNPTG failed to bind at least the lysosomal enzymes ASA, cathepsin D, β-hexosaminidase, and β-glucuronidase.

Discussion

In the present study the importance of the γ-subunit of the GlcNAc-1-phosphotransferase (GNPTG) for the binding of lysosomal enzymes was analyzed. The GlcNAc-1-phosphotransferase complex have to exhibit three binding sites for i) the phosphate donor UDP-GlcNAc, ii) the high-mannose type oligosaccharides on lysosomal enzymes that have to be equipped with M6P residues, and iii) the specific binding site for lysosomal proteins. Due to the similarities to bacterial capsule biosynthesis proteins it has been proposed that the N-terminal domain of the α-subunit comprises the nucleotide sugar binding site [6]. Both α- and β-subunits exhibit catalytic activity sufficient for phosphorylation of small acceptor molecules such as α-methyl mannoside [9]. The transfer of GlcNAc-1-phosphate to lysosomal enzymes as acceptors, however, was shown to require the presence of intact γ-subunits [22, 23]. From these data it has been concluded that the γ-subunit (GNPTG) of

the GlcNAc-1-phosphotransferase complex is responsible for the recognition of lysosomal enzymes [1].

To determine the role of GNPTG in recognition and binding of lysosomal enzymes, two complementary approaches were utilized. First, surface plasmon resonance spectrometry showed that immobilized GNPTG did not bind the purified lysosomal enzyme ASA. Additionally, the immobilized ASA failed to interact with GNPTG. In the second experimental approach we examined whether the overexpression of the soluble γ-subunit is capable to compete with the endogenous GlcNAc-1-phosphotransferase complex *in vivo* during the process of phosphorylation of lysosomal proteins. If GNPTG would function in recognition of lysosomal hydrolases, its overexpression should impair sorting which subsequently results in hypersecretion of newly synthesized lysosomal enzymes into media. Because we have neither observed an increase in the secretion of metabolically labelled cathepsin D nor in the activities of three lysosomal enzymes in the media of GNPTG overexpressing cells, we hypothesize that GNPTG lacks the capability to bind to a conformation-dependent recognition domain present on the surface of many different lysosomal enzymes [24-28]. This hypothesis is supported by our previous studies demonstrating that no lysosomal enzymes could be eluted from a GNPTG affinity matrix [6]. Finally, using brain tissues of mice deficient for GNPTG Kornfeld and colleagues showed recently [29] that the modification of lysosomal enzymes with M6P residues was not generally prevented but that certain hydrolases, e.g. α-mannosidase and β-glucuronidase, were phosphorylated considerably better than others such as β-mannosidase and β-galactosidase. We cannot, however, exclude that GNPTG recognizes lysosomal enzymes only when it is associated with GNPTAB. Such a function would neither be detected in the BIAcore experiments nor in the GNPTG overexpression experiments.

The analysis of fibroblasts of three MLIII patients with mutations in the *GNPTG* gene in this study, however, has provided new data on regulatory functions of GNPTG in the

process of the formation of the M6P recognition marker. In all three MLIII cell lines the relative *GNPTG* mRNA levels were reduced between 25 and 60%. The mutant GNPTG proteins of at least patient 2 (p.N116del [15] and patient 3 (p.K207IfsX7; Fig. 1A) could be detected by Western blotting and appeared to be resistant to ER-associated degradation processes. In these fibroblasts the relative mRNA expression of the genes encoding the α/β-subunits precursors of GlcNAc-1-phosphotransferase (*GNPTAB*) and the phosphodiesterase (*NAGPA*) were found to be upregulated. While the lack of specific antibodies does not allow confirming the upregulation of GNPTAB and NAGPA on protein level the data suggest that compensatory mechanisms might prevent the complete missorting of newly synthesized lysosomal enzymes. Similarly, the overexpression of GNPTG in COS7 cells appears to induce a down-regulation of GNPTAB (Fig. 3B). The compensatory mechanisms of *GNPTG/GNPTAB* mRNA expression and their role in the fine regulation of the formation of the M6P recognition marker are unclear and require further studies.

The novel mutation identified in MLIII gamma patient 3 leads to a frameshift and premature termination of translation, p.K207IfsX7. A total of eight distinct mutations in *GNPTG* have been described in MLIII gamma patients of different ethnic background [8, 14, 15]. The detailed biochemical and molecular analysis of fibroblasts of these patients is essential and represents important and unique sources for investigating the mechanism of phosphorylation of lysosomal enzymes and functional correlations to the genotype.

Together, the data suggest evidence that the γ-subunit of GlcNAc-1-phosphotransferase is not involved in binding of newly synthesized lysosomal enzymes during the phosphorylation of their oligosaccharide chains. In fibroblasts of patients with MLIII gamma the expression of α/β-precursor subunits of GlcNAc-1-phosphotransferase and the uncovering enzyme appears to be altered in a compensatory manner through yet unknown mechanisms.

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Figure legends

Fig. 1. GNPTG expression in fibroblasts of MLIII gamma patient 3. Western blot analysis of GNPTG in control (Co) and MLIII gamma patient 3 (P3) fibroblasts. The positions of molecular mass markers are indicated. The content of β-tubulin in the samples served as control for equal loading (A). GlcNAc-1-phosphotransferase and phosphodiesterase gene expression in MLIII gamma fibroblasts. The relative levels of *GNPTG, GNPTAB*, and *NAGPA* mRNA expression were determined in fibroblasts of MLIII gamma patients by real-time PCR normalized to *β-actin* expression. The relative mRNA expression in control fibroblasts was set 1. Analysis of four control and fibroblasts of MLIII patients 1, 2 and 3 are the mean of triplicate PCRs obtained from two independent RNA preparations. Data are expressed as the mean fold change in respective gene expression ± SD (B).

Fig. 2. BIAcore binding analysis between GNPTG and arylsulfatase A. GNPTG was immobilized to the sensor chip and arylsulfatase A (ASA) passed over the surface at the indicated concentrations (A). ASA was immobilized to the sensor chip and the binding of soluble GNPTG was analyzed (B).

Fig. 3. Stable overexpression of GNPTG. Western blot analysis of GNPTG in extracts of non-transfected (-) and GNPTG expressing (+) COS7 cells. The positions of molecular mass markers are indicated. Equal loading is demonstrated by β-tubulin immunostaining (A). The relative *GNPTG, GNPTAB* and *NAGPA* mRNA level were determined in non-transfected and COS7 cells stably expressing GNPTG by real-time PCR and normalized to *β-actin* mRNA expression. Data are the mean of triplicate PCRs obtained from three RNA preparations and expressed as the fold change ± SD (B).

Fig. 4. Biosynthesis and sorting of the lysosomal enzyme cathepsin D in GNPTG overexpressing cells. Non transfected (-) and *GNPTG* (+) stably transfected cells were labelled with [^{35}S]-methionine for 1 hour and either harvested (-) or chased (+) for 4 hours, followed by immunoprecipitation of cathepsin D from cell extracts and media, separated by SDS-PAGE (10 % acrylamide), and visualized by fluorography. The precursor and mature form of cathepsin D and the positions of molecular mass markers in kDa are indicated. The amounts of secreted cathepsin D precursors were determined by densitometry and are expressed as percentage of total synthesized cathepsin D. A representative fluorograph out of three independent experiments is shown.

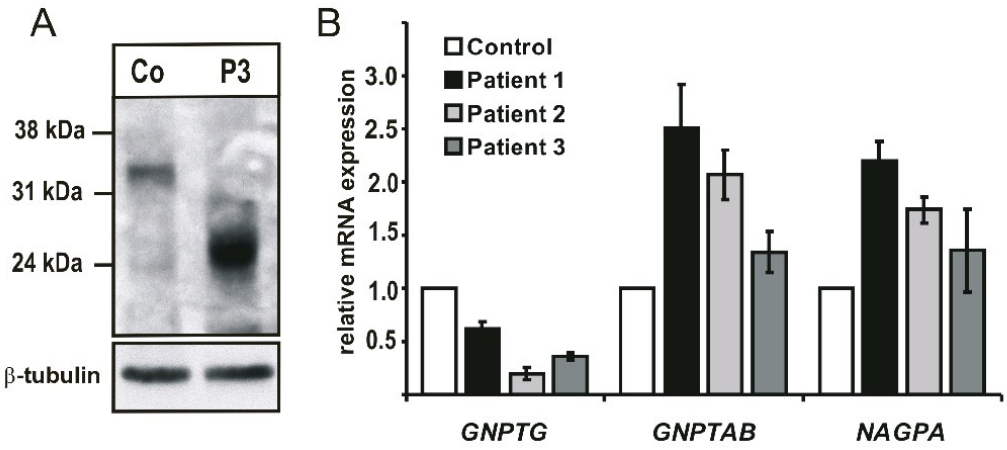

Figure 1
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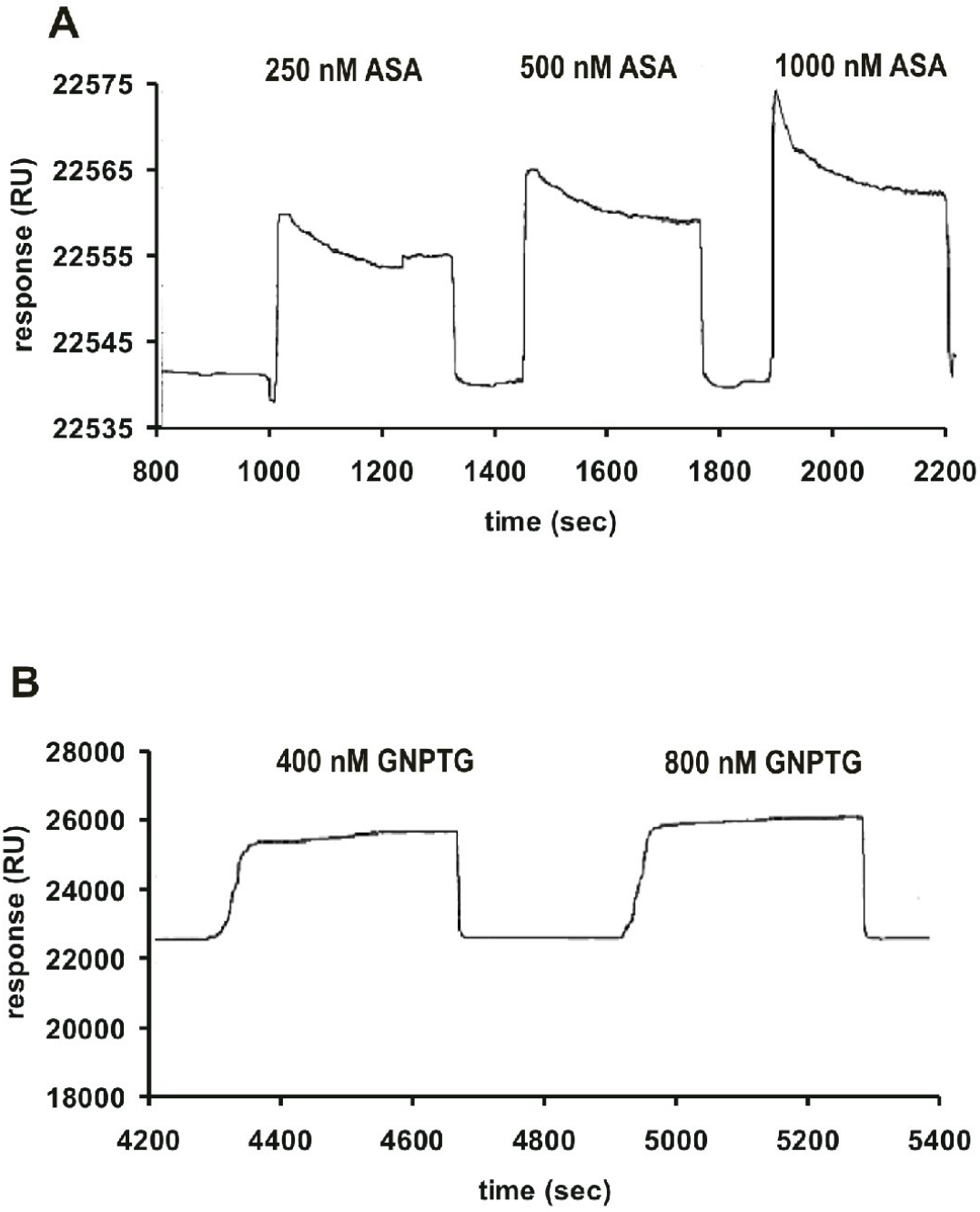

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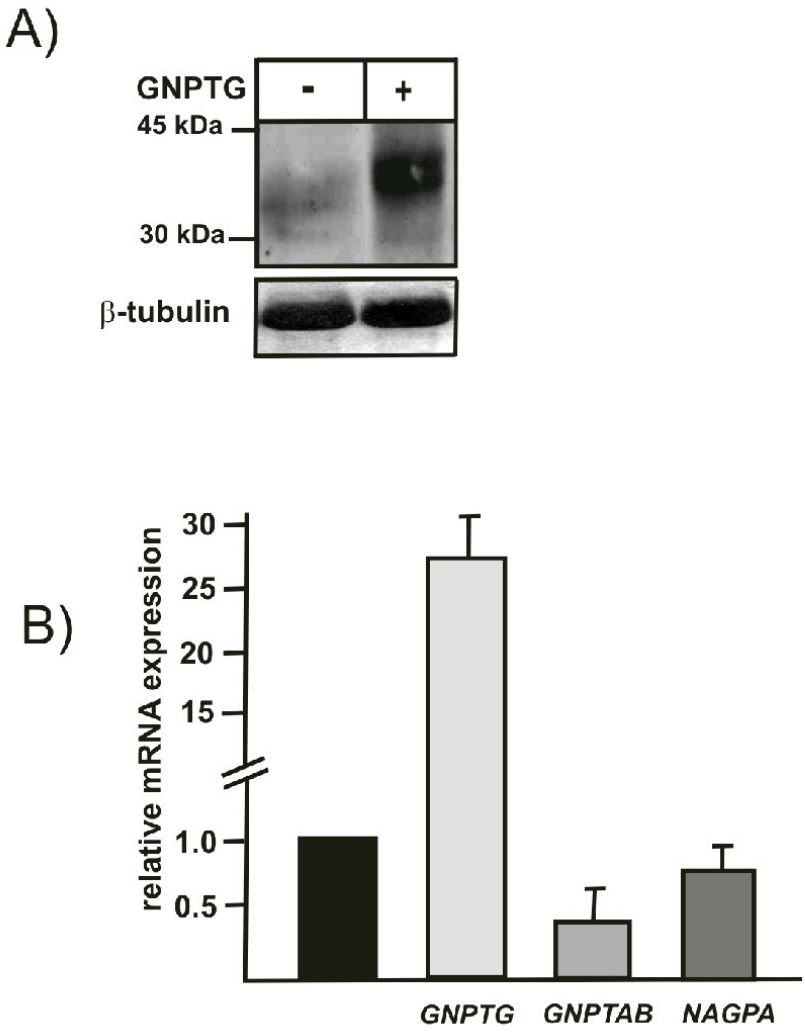

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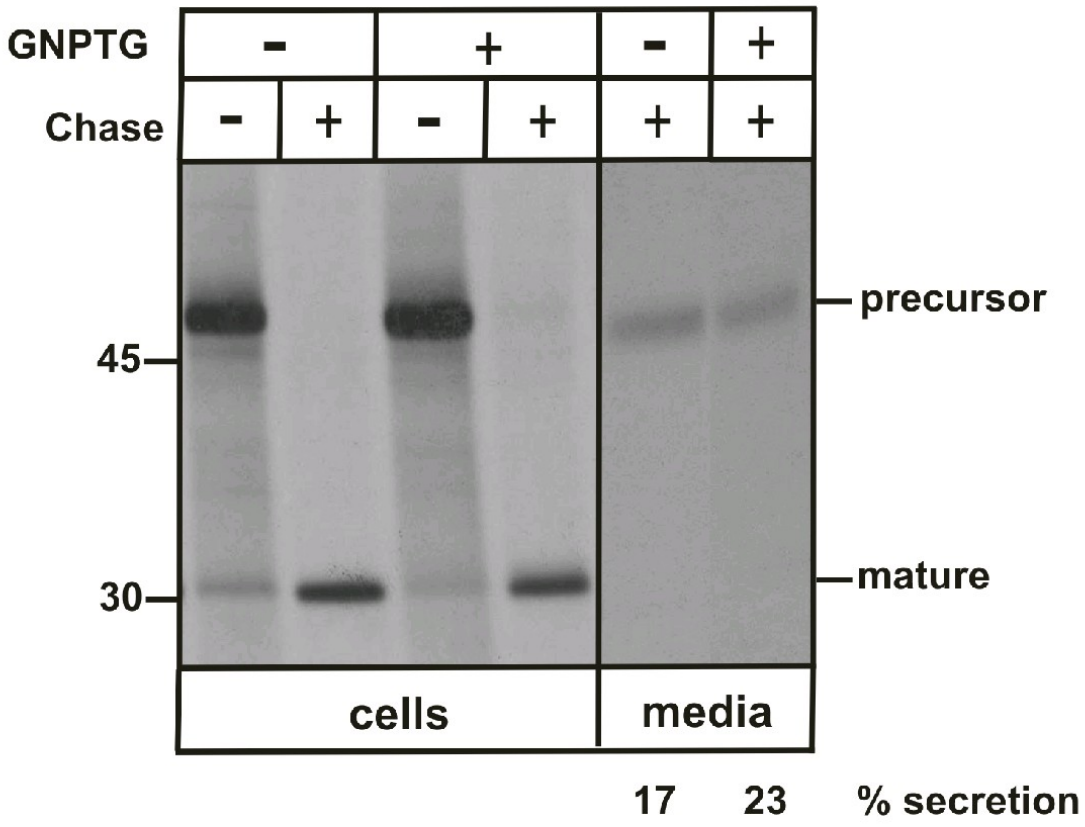
Figure 3



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