Rapid Publication

Missense Mutations in N-Acetylglucosamine-1-Phosphotransferase α/β Subunit Gene in a Patient With Mucolipidosis III and a Mild Clinical Phenotype

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Mucolipidosis type III (ML III, pseudo-Hurler polydystrophy), an autosomal recessive inherited disorder of lysosomal enzyme targeting is due to a defective N-acetylglucosamine 1-phosphotransferase (phosphotransferase) activity and leads to the impaired formation of mannose 6-phosphate markers in soluble lysosomal enzymes following by their increased excretion into the serum. Mutations in the phosphotransferase γ subunit gene (GNPTAG) have been reported to be responsible for ML III. Here we report on a 14-year-old adolescent with a mild clinical phenotype of ML III. He presented with progressive joint stiffness and swelling. Urinary oligosaccharide and mucopolysaccharide excretion was normal. Lysosomal enzyme activities were significantly elevated in the serum and decreased in cultured fibroblasts. Impaired trafficking of the lysosomal protease cathepsin D (CtsD) was confirmed by metabolic labeling of the patient's fibroblasts. Neither mutations in the GNPTAG gene nor alterations in the GNPTAG mRNA level were detected whereas the steady state concentration of the 97 kDa GNPTAG dimer was reduced. Most importantly, the patient is homozygous for a pathogenic nucleotide substitution and a polymorphism in the phosphotransferase α/β subunit gene (GNPTA). The data indicate that defects in genes other than GNPTAG can be linked to ML III contributing to the variability of the phenotype.

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KEY WORDS: mucolipidosis type III; phosphotransferase; pseudo-Hurler polydystrophy; cathepsin D; lysosomal storage disorders

INTRODUCTION

Mucolipidosis type III (ML III, pseudo-Hurler polydystrophy, MIM 252605) is an autosomal recessive disorder of lysosomal enzyme targeting caused by a deficiency of UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine 1-phosphotransferase (phosphotransferase, GNPTA, MIM 607840). Phosphotransferase catalyzes the formation of mannose 6-phosphate (M6P) markers on high mannose type oligosaccharides in the Golgi apparatus. M6P residues are required to bind to the M6P receptors (MPR), which mediate the vesicular transport of lysosomal enzymes to the endosomal/prenysosomal compartment. Due to the low pH in these organelles, the lysosomal enzyme–MPR complex dissociates and then the enzyme is delivered to the lysosome. Between 5% and 20% of newly synthesized lysosomal enzymes escape the binding to the MPR in the Golgi apparatus and are secreted

[Storch and Braulke, 2005]. The phosphotransferase has been reported to be composed of three subunits α, β, and γ [Bao et al., 1996] encoded by two genes [Kornfeld and Sly, 2001]. It is believed that the α and/or the β subunit of the phosphotransferase contain the catalytic portion of the enzyme and that the γ subunit functions in recognition of the lysosomal enzymes [Raas-Rothschild et al., 2000]. The gene for the γ subunit (GNPTAG, MIM 607838) is affected in patients with ML III [Raas-Rothschild et al., 2000, 2004; Tiede et al., 2004]. In these patients, phosphotransferase activity is reduced and soluble lysosomal enzymes are excreted mainly in the body fluids and serum [Reitman et al., 1981]. Clinically ML III is characterized by restricted joint mobility, skeletal dysplasia, and short stature. Mildly coarsened facial features and thickening of the skin have been described. Cardiac valvular disease and corneal clouding may also occur. Half of the reported patients show learning disabilities or mental retardation [Spranger and Wiedemann, 1970; Reitman et al., 1981; Raas-Rothschild et al., 2000, 2004; Kornfeld and Sly, 2001; Tiede et al., 2004].

We report on a 14-year-old adolescent with a mild clinical phenotype of ML III, who presented with progressive joint stiffness and elevated lysosomal enzyme activities in the serum. Pulse-chase experiments confirmed the missorting of newly synthesized lysosomal enzymes in fibroblasts of the patient. Although the expression of the GNPTAG protein was reduced and its cellular distribution altered, neither mutations in the GNPTAG gene nor changes in the GPNTAG mRNA level have been detected. In contrast, the patient is homozygous for a pathogenic missense mutation in the GNPTA gene encoding the α/β subunits of phosphotransferase, indicating that different gene defects may contribute to the clinical variety of ML III.

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MATERIALS AND METHODS

Materials

[^35]S-Methionine and rainbow-colored protein molecular mass marker were purchased from Amersham Biosciences (Freiburg, Germany). Mannose 6-phosphate (M6P), protein A-agarose, protease inhibitor cocktail (P-2714), and glutathione-agarose were obtained from Sigma (Deisenhofen, Germany). For reverse transcription of mRNA the GeneAmp RNA PCR kit was purchased from Roche (Branchburg, NJ). Oligonucleotides used for sequencing were synthesized by MWG Biotech (Munich, Germany). For real-time PCR experiments the LightCycler-FastStart DNA Master SYBR Green I kit and the LightCycler Instrument were used (Roche, Mannheim, Germany). For reverse transcription of mRNA the GeneAmp RNA PCR kit was purchased from Roche (Branchburg, NJ). Oligonucleotides used for sequencing were synthesized by MWG Biotech (Munich, Germany). For real-time PCR experiments the LightCycler-FastStart DNA Master SYBR Green I kit and the LightCycler Instrument were used (Roche, Mannheim, Germany). Fetal calf serum (FCS), Dulbecco’s modified minimal essential medium (DMEM), RPMI-medium and penicillin/streptomycin were from Gibco BRL, Life Technologies (Karlsruhe, Germany).

Patient and Sample Isolation

The patient (patient 10) is the first child of non-consanguinous German parents. His 7- and 12-year-old sisters are healthy. He first presented at 2 years of age with recurrent swelling and progressive stiffness in his wrists and finger joints. As the disease progressed there was further swelling and stiffness, which included the knees, elbows, shoulders, and spine. Later, because of carpal tunnel syndrome hand surgery was performed successfully on both hands. Now at 14 years of age no signs of mental retardation. The patient’s weight falls within the 25–50th centile, his height in the 50–75th centile, head circumference falls in the 97th centile. Ophthalmological examinations did not show any corneal clouding. Abdominal ultrasound found no enlargement of the liver or spleen, and kidneys were normal. Echocardiography and lung function testing was normal. Spinal skoliosis, kyphosis, and stiffness as well as reduced joint mobility of shoulders are present (Fig. 1b). Physical examination showed a stiff gait, finger contractures but normal tendon reflexes (Fig. 1c). The examination found reduced joint motility, most markedly in the spine, lumbar region, and shoulders. The spine had kyphotic and kyphotic deformities. Radiographic examination and MRI investigations of the spine and pelvis showed dysostosis multiplex (Fig. 2) and spondylolisthesis of L5/S1.

The activity of several lysosomal enzymes were significantly elevated in the serum (Table I) and decreased in cultured fibroblasts (not shown). The pattern of urinary oligosaccharides and mucopolysaccharides was normal. The clinical and biochemical findings were compatible with the diagnosis of ML III. Genomic DNA was isolated from fibroblasts and blood using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Total RNA was isolated by means of guanidine isothiocyanate extraction, caesium chloride density-gradient ultracentrifugation and ethanol precipitation [Chirgwin et al., 1979].

Cell Culture

Fibroblasts of ML III patient 10 and controls were cultured in RPMI medium containing 20% FCS and penicillin/streptomycin and incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

Fig. 1. Patient at age 14. a: Patient does not have coarse facial features. b: Reduced joint mobility in the shoulders. c: Finger contractures.

Fig. 2. Radiograph of the pelvis and thoraco-lumbar spine of the ML III (mucolipidosis type III) patient. a: Flattened femoral epiphysis and irregular, hypoplastic acetabula. b and c: Skoliosis and vertebral deformity with platyspondyia.
Antibodies

Polyclonal antibodies against human GNPTAG were raised commercially by EUROGENTEC (Cologne, Germany) and affinity-purified as described previously [Tiede et al., 2004]. Polyclonal antibodies against human cathepsin D (CtsD) were kindly provided by K. von Figura (University of Göttingen, Germany). Monoclonal antibody against GM130 was purchased from Transduction Laboratories (Lexington, KY). Secondary antibodies conjugated to horseradish peroxidase for Western blot analysis and fluorochrome conjugated antibodies used for immunofluorescence microscopy were purchased from Dianova (Hamburg, Germany).

Real-Time RT-PCR

RNA (1 µg) was reversed transcribed in a total of 20 µl using the Perkin-Elmer RNA PCR kit (Branchburg, NJ). For amplification of GNPTAG and β-actin, parts of the cDNA products (2 µl) were subjected to real-time PCR analysis. GNPTAG cDNA was amplified with primers PT for (5'-GCAGCGGCAGTGGGACCAAGGT-3') and PTrev (5'-TCCACCTGTCGCTCCACCA-3'). β-Actin amplification, used as internal control, was carried out as described [Heine et al., 2002].

Sequencing and Mutation Analysis

The 11 exons of the GNPTAG gene were amplified by PCR as described [Tiede et al., 2004]. After separation of the PCR reaction mix by 2% agarose gel electrophoresis the products were excised and purified (Qiagen). Sequence reactions were performed with BigDye Terminator Reaction Kit (PE Applied Biosystems, Foster City, CA) 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 GNPTAG sequences (GenBank: BC014592). Nucleotide numbering was referred to the ATG start codon, using the A as + 1.

Exon specific PCR amplification and sequencing of GNPTA encoding the α/β subunits of the phosphotransferase [Tiede et al., submitted] was done by using primer sets specific for exon 10 (10 for 5'-ACAGCTTCTCTACATTTCTTCTT-3' and 10rev 5'-GCTAAGTGAATTTCCAGCTAG-3') and exon 13 (13for 5'-ATCATGTTTCTTTTCT-3' and 13rev 5'-AATGAAACCATGTAAGAAAG-3').

Immunofluorescence Microscopy

For double immunofluorescence microscopy ML III and control fibroblasts were cultured for 14 days on glass cover slips in a 24-well plate. Cells were washed and fixed with methanol on ice for 5 min, washed again and blocked with phosphate buffered saline, pH 7.4 (PBS) containing 1% bovine serum albumin (PBS–BSA). Cells were incubated using affinity-purified GNPTAG IgG and anti-GM130 (1:100) in PBS–BSA for 60 min at room temperature. Incubation with secondary antibodies was performed at room temperature for 60 min using anti-mouse Cy3 (1:2,000) and anti-rabbit FITC (1:100) in PBS–BSA. After washing with PBS–BSA, cells were incubated with secondary antibodies conjugated to the fluorochromes Cy3 and FITC for 60 min at room temperature as indicated. After washing with PBS, the cover slips were mounted in fluorescent mounting medium (DAKO, Glostrup, Denmark), processed for immunofluorescence microscopy and analyzed using a Zeiss Axiosvert S100 microscope (Carl Zeiss, Göttingen, Germany) equipped with an Olympus dp50 digital camera.

Other Methods

Metabolic labeling of cells with [35S]-methionine followed by immunoprecipitation of CtsD, SDS–PAGE, and fluorography
were described previously [Braulke et al., 1990] GNPTAG Western blot analysis was performed as described [Tiede et al., 2004]. The activities of lysosomal enzymes in serum-free media and extracts of fibroblasts were determined as described previously [Porter et al., 1969; Hall et al., 1973; Gehler et al., 1974, 1976; von Figura, 1978].

RESULTS

Pulse/chase experiments with [³⁵S]-methionine and immunoprecipitation of the lysosomal protease CtsD were carried out with the patient's and control fibroblasts. In control cells, the majority of newly synthesized CtsD was transported to the lysosome and proteolytically processed to the 31 kDa mature form (Fig. 3). Densitometric evaluation of the fluorogram revealed that 16% of the newly synthesized CtsD was immunoprecipitated from the medium as a 53 kDa precursor form. In the patient's cells the 31 kDa mature form was not observed and 86% of the newly synthesized CtsD precursor form was isolated from the medium. This data suggests that the newly synthesized lysosomal enzymes in the patient lack M6P residues able to bind to the MPR, preventing efficient sorting in the Golgi apparatus.

To examine whether the lack of M6P residues is due to a defective γ subunit of phosphotransferase (GNPTAG), the genomic DNA of the patient was sequenced directly. No mutations were found in the GNPTAG gene (including the intron structures 1, 4, 5, 6, 8, and 10). Quantification of the GNPTAG mRNA level by real time PCR showed no variation in the patient's fibroblasts compared to the control or cells from other ML III patients (Table II). When the expression of GNPTAG was examined on the protein level by Western blotting, a 97 kDa disulfide-linked GNPTAG dimer was detected in the control cells (Fig. 4). In extracts of the patient's fibroblasts, the amount of the 97 kDa dimer was greatly reduced and was accompanied by the appearance of a 40 kDa immunoreactive polypeptide which was absent from the control fibroblasts. Double immunofluorescence microscopy and quantitation of each ten cells showed that approximately 64% of the GNPTAG protein was colocalized with the Golgi marker GM130 in the control fibroblasts and one third was observed as a punctual vesicular staining throughout the cell (Fig. 5). In fibroblasts from the patient the colocalization of GNPTAG and GM130 was weak accounting approximately 10% of total GNPTAG immunoreactivity.

When the amplified exons encoding the α/β subunits of the phosphotransferase were sequenced using genomic DNA from cells of the patient, two single nucleotide substitutions in exon 10 (c.1220A>C) and 13 (c.1895C>G) were identified leading to changes of Asp to Ala (p.D407A) and Ala to Gly (p.A663G), respectively. To examine possible non-pathogenic polymorphisms among the newly identified amino acid exchanges, the respective exons of the phosphotransferase gene of 100 unaffected individuals, and of the parents of the patient were amplified and sequenced. The D407A mutation appears to be pathogenic as it was homozygously present in the patient, and heterozygously expressed in the alleles of the patient's parents but not in the 200 normal alleles. The sequence variant A663G was shown to be polymorphic as it was found in 5% of normal alleles. Whereas the patient was homozygous for the A663G polymorphism, his parents express this substitution heterozygously.

These data indicate that the GNPTAG gene is not defective in the ML III patient but the mutations in the GNPTAG gene alter the α/β subunits and affect secondary both the intracel-

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**TABLE II. Expression of GNPTAG in Fibroblasts of the ML III Patient**

<table>
<thead>
<tr>
<th>Fibroblasts</th>
<th>Days in culture</th>
<th>GNPTAG*a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>10.0 ± 0.1</td>
</tr>
<tr>
<td>ML III</td>
<td>15</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>Pat. 10</td>
<td>14</td>
<td>9.7 ± 0.2</td>
</tr>
</tbody>
</table>

*a,GNPTAG mRNA/β-actin mRNA ± SD.
bMean of four independent experiments with two to three different RNA preparations.

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**Fig. 3. Sorting of newly synthesized cathepsin D (CtsD) in fibroblasts of the ML III patient.** Fibroblasts from the control (Co) and the ML III patient (Pat. 10) were labeled for 60 min with [³⁵S]-methionine (150 μCi/ml) and chased for 6 hr. CtsD was immunoprecipitated from cell extracts and media, and samples were analyzed by SDS–PAGE (10% acrylamide) and fluorography. The percentage of CtsD secreted into the medium is given. The position of molecular mass marker proteins, precursor (p), intermediate (i), and mature (m) forms of CtsD are indicated.

**Fig. 4. Expression of GNPTAG in fibroblasts of patient 10.** Cell extracts of the control (Co) and the ML III patient (Pat. 10) were separated by SDS-PAGE under non-reducing conditions, transferred onto nitrocellulose and analyzed by GNPTAG Western blotting. The positions of the molecular mass markers (in kDa) are indicated.
cular distribution, preferentially in non-Golgi structures, and the steady-state expression of the γ subunit.

**DISCUSSION**

In this study, we report on a male 14-year-old patient with mild clinical, radiographic, and biochemical findings of ML III including joint stiffness, dysostosis multiplex and elevated levels of lysosomal enzymes in the serum. Other features such as mental retardation, corneal clouding, and valvular heart disease were not present.

The elevation of serum activities of lysosomal enzymes are enzyme-specific and differ greatly in all ML III patients [Kelly et al., 1975; Ward et al., 1993; Raas-Rothschild et al., 2004; Tiede et al., 2004]. A genotype–phenotype correlation has not been established yet in ML III because of the small number of patients, and because of possible epigenetic influences as suggested by marked intrafamilial variability, and a more severe clinical expression in males than in their affected sisters [Melhem et al., 1973; Brik et al., 1993; Tylki-Szymanska et al., 2002]. It is believed that the γ subunit of the phosphotransferase (GNPTAG) plays a major role in ML III. Seven different mutations in nine affected families have so far been identified including: missense, frameshift, and splice mutations [Raas-Rothschild et al., 2000, 2004; Tiede et al., 2004]. Although the reduced level of the GNPTAG protein in fibroblasts of the patient presented here suggests mutations in the GNPTAG gene, sequencing of GNPTAG failed to detect a mutation. Furthermore, normal levels of GNPTAG mRNA and the appearance of a 40 kDa GNPTAG immunoreactive protein band in cell extracts of the patient suggest that post-translational modifications, most likely degradation, rather than mRNA instability may explain the reduced GNPTAG protein level. However, two mutations in the GNPTA gene encoding the α/β subunits of phosphotransferase have been identified, one missense mutation (D407A) and one polymorphism (A662G). The D407A mutation can be considered to be pathological because it was not found in 200 normal control alleles. The substitution of the acidic aspartate residue with a small, non-polar alanine residue (D407A) destroys a highly conserved ND^\text{D^\text{D}} motif in a domain related to N-terminal acetylglucosamine biosynthesis proteins [Tiede et al., submitted]. It is likely that this domain contains the UDP-N-acetylglucosamine binding site. During the preparation of the manuscript, Kornfeld et al. reported on a homozygous mutation in the GNPTA gene in an adult onset ML III patient [Steet et al., 2005]. The nucleotide substitution resulted in aberrant splicing and skipping of exon 7. The predicted loss of the amino acid residues 213–257 are located in an inserted GNPTA-specific module in the N-terminal conserved domain. In contrast to the mutations in GNPTA described in the two ML III patients, the mutations identified so far in patients with the more severe phenotype of ML II were found in the more distal exons 13–20 [Tiede et al., submitted]. The functional significance of the different domains in the α/β subunits of the phosphotransferase and the structural requirements for the interaction between the three subunits remain to be investigated. Preliminary results suggest that the number of endogenous α/β subunits of the phosphotransferase is limiting for the cellular retention of γ subunits because overexpression of the γ subunit in BHK cells results in the secretion of varying amounts of γ subunits in the medium [Tiede and Braulke, unpublished results]. Thus, it is likely that the mutation D407A reported here lead not only to alterations in the activity of phosphotransferase but might also affect the stability of the enzyme as has been reported, for example, on lysosomal sulfamidase [Muschol et al., 2004], and secondary impair the assembly and cellular distribution of the γ subunit.

**ACKNOWLEDGMENTS**

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