Biochimica et Biophysica Acta 1822 (2012) 1137–1146

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

# Lysosomal di-N-acetylchitobiase-deficient mouse tissues accumulate Man2GlcNAc2 and Man3GlcNAc2

Emanuele Persichetti [a], Katharina Klein [b], Silvia Paciotti [a], Karine Lecointe [c], Chiara Balducci [a], Sebastian Franken [b], Sandrine Duvet [c], Ulrich Matzner [b], Rita Roberti [d], Dieter Hartmann [e], Volkmar Gieselmann [b], Tommaso Beccari [a,*]

[a] University of Perugia, D.S.E.E.A., Institute of Food Chemistry, Biochemistry, Physiology and Nutrition, 06126 Perugia, Italy
[b] Institut für Biochemie und Molekularbiologie, Nussallee 11, Rheinische Friedrich-Wilhelms-Universität Bonn, 53115 Bonn, Germany
[c] Unité de Glycobiologie Structurale et Fonctionnelle,UMR/CNRS 8576,IFR147, Université des Sciences et Technologies de Lille,F-59655 Villeneuve d'Ascq, France
[d] University of Perugia, Department of Internal Medicine, Laboratory of Biochemistry, via del Giochetto, 06122 Perugia, Italy
[e] Institute of Anatomy, Division of Neuroanatomy, University of Bonn, Nussallee 10, 53115 Bonn, Germany

ARTICLE INFO

*Article history:*
Received 2 December 2011
Received in revised form 1 March 2012
Accepted 12 March 2012
Available online 20 March 2012

*Keywords:*
Lysosomal di-N-acetylchitobiase
Lysosomal storage disease
Knockout mouse
N-linked glycan

ABSTRACT

Most lysosomal storage diseases are caused by defects in genes encoding for acidic hydrolases. Deficiency of an enzyme involved in the catabolic pathway of N-linked glycans leads to the accumulation of the respective substrate and consequently to the onset of a specific storage disorder. Di-N-acetylchitobiase and core specific α1–6mannosidase represent the only exception. In fact, to date no lysosomal disease has been correlated to the deficiency of these enzymes. We generated di-N-acetylchitobiase-deficient mice by gene targeting of the Ctbs gene in murine embryonic stem cells. Accumulation of Man2GlcNAc2 and Man3GlcNAc2 was evaluated in all analyzed tissues and the tetrasaccharide was detected in urines. Multilamellar inclusion bodies reminiscent of polar lipids were present in epithelia of a scattered subset of proximal tubules in the kidney. Less constantly, enlarged Kupffer cells were observed in liver, filled with phagocytic material resembling partly digested red blood cells. These findings confirm an important role for lysosomal di-N-acetylchitobiase in glycans degradation and suggest that its deficiency could be the cause of a not yet described lysosomal storage disease.



## 1. Introduction

During the degradation of glycoproteins with asparagine-linked carbohydrates, the N-acetylglucosamine residue at the reducing end of the N,N′ diacetylchitobiose core of oligosaccharides is hydrolyzed by the lysosomal glycosidase di-N-acetylchitobiase (chitobiase) (EC 3.2.1.-) [1]. This enzyme was first purified and characterized in human liver [2]; human and rat chitobiase cDNAs were cloned [3].

To date, no storage disease has been described in humans or animals as a result of chitobiase deficiency. However, cloning and characterization of the human core-specific lysosomal α1–6mannosidase (MAN2B2) demonstrated that this enzyme is dependent on chitobiase activity [4–7] during the degradation of the Manα1–3[Manα1–6]Manβ1–4GlcNAcβ1–4GlcNAc core structure resulting from the catabolism of N-linked oligosaccharides (Fig. 1, panel A). While lysosomal α-mannosidase (LysMan) can cleave the Manα1–3 from the non-reducing end of this structure, the efficient removal of Manα1–6 residue by α1–6mannosidase requires the trimming of the reducing terminal GlcNAc by chitobiase. These data are confirmed by the accumulation of Manα1–6Manβ1–4GlcNAcβ1–4GlcNAc-Asn in tissues of N-aspartylglucosaminidase-deficient mice [8], a mouse model for the lysosomal disorder aspartylglycosaminuria.

Chitobiase, as well as lysosomal α1–6mannosidase, is expressed only in humans and rodents, whereas alterations in promoter sequence inhibit its expression in ruminants, felines and dogs [9,10]. This difference could explain the variation in the glycan structures accumulated in subjects affected by alpha-mannosidosis, a lysosomal storage disease caused by the genetic deficiency of α-mannosidase, in various species. In cattle, cats and dogs a deficiency of α-mannosidase causes the accumulation of the tri-mannosyl core (Manα1–3[Manα1–6]Manβ1–4-GlcNAcβ1–4GlcNAc) in addition to the extended structures on the α1–3Man branch [11]. In humans and rodents the main stored structure is Manα1–3Manβ1–4GlcNAc, along with other extended products on the α1–3Man branch [6,12,13].

The murine gene encoding the chitobiase (Ctbs) has been recently cloned [14]. The gene spans about 15 kb from the transcription initiation site to the translation stop codon and includes 7 exons, like

* Corresponding author at: Università di Perugia, D.S.E.E.A., Via San Costanzo, 06121 Perugia, Italy. Tel.: +39 075 5857441; fax: +39 075 5857904.
*E-mail address:* tbeccari@unipg.it (T. Beccari).

0925-4439/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2012.03.005

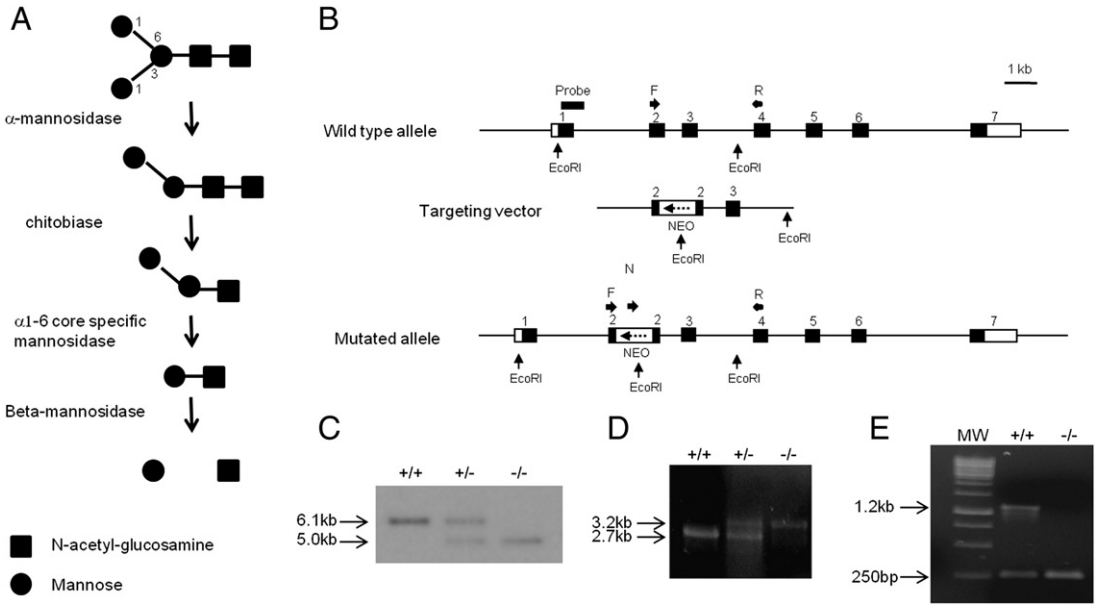


**Fig. 1.** Degradation of the N-linked glycan core and targeted disruption of Ctbs gene. (A) Stepwise trimming of the glycan core derived from N-linked oligosaccharides. The removal of the terminal N-acetyl-glucosamine by chitobiase is essential for the core specific α1–6 mannosidase activity. (B) Schematic representation of the endogenous Ctbs gene, the targeting construct and the disrupted allele. Boxes are the exons. Black parts indicate coding regions, white parts represent non-coding regions. The 5′ probe used for Southern blot is indicated, as well as primers used to screen recombinant ES clones and mouse litters (F, R, N). Arrow within the NEO cassette indicates transcriptional orientation. (C) Southern blot analysis of liver genomic DNA. EcoRI digested DNA of wild type (+/+), heterozygous (+/−) and KO (−/−) mice was separated on an agarose gel, blotted and hybridized with the 5' probe. The 6.1 kb band corresponds to the wild type allele, the 5.0 kb band represents the mutant allele. (D) PCR genotyping of mouse tail DNA. Primers F, R and N were used in the same reaction. 3.2 kb and 2.7 kb bands correspond to mutated and wild type allele, respectively. (E) RT-PCR of kidney RNA. RNA of wild type and CTBS deficient mice was retrotranscribed and PCR amplified. 1.2 kb band represents the main CTBS transcript, obtained with a primer forward on 5′ UTR and a primer reverse on 3′ UTR. 250 bp band corresponds to a fragment of beta-actin cDNA, used as an internal control.

the human CTBS gene [10]. The amino acid sequences of murine and human enzymes show 79% identity and 86% similarity.

In this study, we present the first chitobiase-deficient mouse model. In order to disrupt the Ctbs gene, the open reading frame of the neomycin resistance gene (NEO) was inserted in exon 2. RT PCR, enzyme assay and Western blotting analyses confirmed that no detectable amount of chitobiase was expressed in chitobiase KO mice. Although no overt phenotypical alteration is evident, significant amounts of the Man2GlcNAc2 tetrasaccharide and, to a minor extent, of the Man3GlcNAc2 pentasaccharide accumulate in several KO tissues. Histological analysis reveals typical features of other murine models of lysosomal storage diseases: firstly and more evidently the presence of multilamellar inclusion bodies indicative of polar lipids in epithelial cells of scattered proximal kidney tubules, in addition to enlarged vacuolated Kupffer cells. Preliminary data from lectin screening indicate that mannosyl compounds may accumulate even in those epithelial cells that are still devoid of ultrastructurally visible storage material.

The accumulation of oligosaccharides and the pathological features observed in this mouse model provide the first *in vivo* evidence of the crucial role of chitobiase in glycans degradation. Since the role of chitobiase is the same in mice and humans, this result suggests that the genetic lack of chitobiase could be the cause of a not yet described human lysosomal storage disease.

## 2. Materials and methods

### 2.1. Construction of the targeting vector

A 5.4 kb XbaI fragment obtained from the Mouse Genomic λ FIX II library (Stratagene) and corresponding to the region from intron 1 to intron 3 of mouse ctbs gene was cloned in pBlueScript SK (Stratagene). A BamHI site was created into exon 2 by site directed mutagenesis. This site was used to subclone the NEO gene from pBSK-NEO vector. Orientation of this fragment was confirmed by restriction analysis. The entire cassette was sequenced.

### 2.2. Generation of chitobiase knockout mice

200 micrograms of the targeting vector were linearized with NotI and introduced into 3×107 HM-1 murine embryonic stem (ES) cells [15] by electroporation (Bio-Rad Gene Pulser II; 0.8 kV, 3 μF). The ES cells were maintained on feeder layers under the selective pressure of G418 containing medium (Sigma-Aldrich) for 8 days. ES cell clones were picked, expanded and screened by PCR for homologous recombination. Targeted clones were reconfirmed by Southern blot analysis after digestion with EcoRI and hybridization with a 5' external probe. ES cells from two targeted clones were microinjected into blastocysts from C57BL/6 N (Charles River) mice and transferred to pseudopregnant recipient females. A total of 10 chimerae were born, with a high contribution of agouti coat colour. The chimeric animals were bred with C57BL/6 N mice, and agouti pups were screened for germ line transmission of the mutant allele. The genotypes of the offspring of these matings and all subsequent offspring were determined by PCR on DNA from tail biopsy specimens.

The following primers were used: ctbsFOR (primer F in Fig. 1), 5′-ATGACTGGTCACAGATTACAACTG-3′; ctbsREV (primer R), 5′-ATGGTG ATCAACAAGGATGAGTATC-3′; NEOREV (primer N), 5′-ACTTCGCCCA ATAGCAGCCAG-3′. PCR conditions were the following: denaturation 98 °C for 3 min, followed by 35 cycles of 30 sec at 98 °C, 30 sec at 64 °C and 90 sec at 72 °C. The PCR resulted in fragments of 2700 bp for the wild-type and 3200 bp for the mutant fragment.

Mice used in this study were housed under standard conditions in a 12-h light–dark cycle with food and water ad libitum. All experiments were carried out in accordance with local and state regulations for research with animals.