FEBS Letters 588 (2014) 746–751

journal homepage: www.FEBSLetters.org

Human chitotriosidase CHIT1 cross reacts with mammalian-like substrates

Tanja Larsen [a,1], Yayoi Yoshimura [b,1], Bjørn G.R. Voldborg [c,2], Giuseppe Cazzamali [c], Nicolai V. Bovin [d], Ulrika Westerlind [e], Monica M. Palcic [b], Jørgen J. Leisner [a,*]

[a] Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Grønnegaardsvej 15, 1870 Frederiksberg C, Denmark
[b] Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-1799 Copenhagen V, Denmark
[c] Novo Nordisk Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3b, DK-2200 Copenhagen N, Denmark
[d] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow 117997, Russian Federation
[e] Gesellschaft zur Förderung der Analytischen Wissenschaften e.V., ISAS – Leibniz Institute for Analytical Sciences, Otto-Hahn-Str. 6b, D-44227 Dortmund, Germany

ARTICLE INFO

Article history:
Received 26 October 2013
Revised 27 December 2013
Accepted 27 December 2013
Available online 23 January 2014

Edited by Stuart Ferguson

Keywords:
Chitinases
Family 18 glycosidases
Kinetics
GlcNAc-containing substrates
Glycoconjugates
LacNAc
LacdiNAc

ABSTRACT

Humans do not synthesize chitin, yet they produce a number of active and inactive chitinases. One of the active enzymes is chitotriosidase whose serum levels are elevated in a number of diseases such as Gaucher's disease and upon fungal infection. Since the biological role of chitotriosidase in disease pathogenesis is not understood we screened a panel of mammalian GlcNAc-containing glycoconjugates as alternate substrates. LacNAc and LacdiNAc-terminating substrates are hydrolyzed, the latter with a turnover comparable to that of *p*NP-chitotriose. Glycolipids or glycoproteins with LacNAc and LacdiNAc represent potential chitinase substrates and the subsequent alteration of glycosylation pattern could be a factor in disease pathogenesis.

1. Introduction

Chitin, a linear polymer of β1,4 linked N-acetylglucosamine (GlcNAc) is found in insects, crustaceans and most fungi, but not in plants, vertebrates or prokaryotes. Chitinases that hydrolyze chitin are ubiquitous enzymes produced by a wide variety of organisms, including some that do not contact or produce chitin [1]. These include humans which produce two catalytically active chitinases, chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase) and four catalytically inactive chitinase-like proteins or chitolectins with mutations in the putative active site [2–5]. Human chitinases and chitolectins are all members of CAZy glycoside hydrolase family 18 [6]. The catalytically active enzymes CHIT1 and AMCase are both 50 kDa proteins, comprised of a 39 kDa catalytic region and C-terminal CBM 14 chitin binding module [3,7].

CHIT1 is produced in mature macrophages, neutrophils, Gaucher's cells and lung macrophages [4,5,7] and CHIT1 plasma levels are elevated in pathological conditions including Gauchers disease where it is secreted as the active full-length 50 kDa form [8]. An active proteolyzed 39 kDa form is found in lysosomes. The biological basis for the roles of chitotriosidase in human disease remains unclear since no cellular targets have been identified. In this study we survey a panel of GlcNAc containing glycoconjugates as alternate substrates for CHIT1. The structures are fluorescently labelled conjugates to facilitate screening and they contain terminal LacNAc (Galβ1-4GlcNAc), LacdiNAc (GalNAcβ1-4GlcNAc) or Type 1 (Galβ1-3GlcNAc) motifs (Fig. 1 and Supplementary Fig. 1). Several of these conjugates were hydrolyzed including LacdiNAc at a comparable rate to that of *p*NP-chitotriose. Hydrolysis of cellular glycoconjugates with these terminal structures might be associated with chitotriosidase pathologies.

Abbreviations: CHIT1, chitotriosidase; AMCase, acidic mammalian chitinase

* Corresponding author. Fax: +45 35332757.
E-mail address: jjl@sund.ku.dk (J.J. Leisner).

[1] These authors contributed equally to this work.

[2] Present address: Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kogle Alle 6, DK-2970, Denmark.

http://dx.doi.org/10.1016/j.febslet.2013.12.035

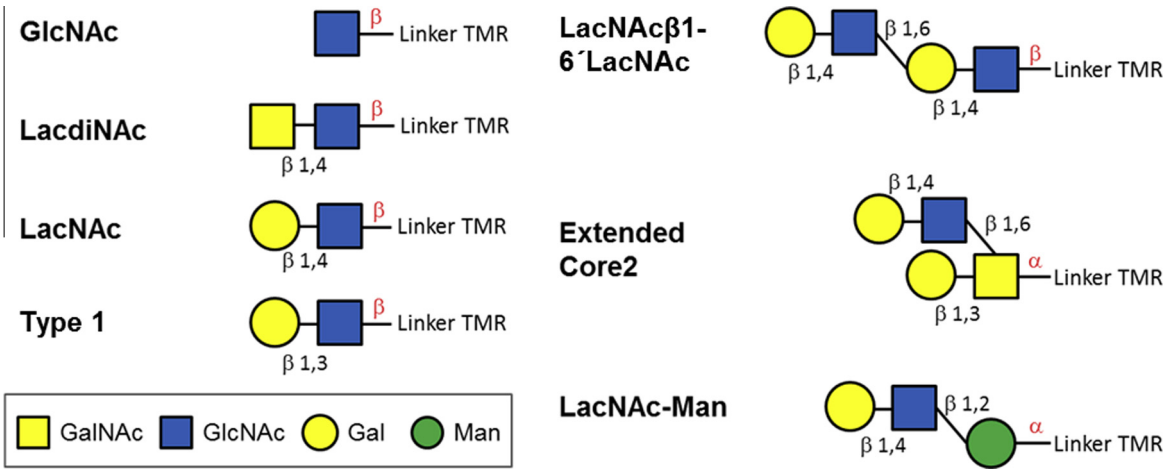

Fig. 1. Fluorescent substrates used in this study. Their exact structures are shown in Supplementary Fig. 1.

2. Materials and methods

2.1. Production of CHIT1 in HEK293 EBNA cells

Full-length CHIT1 cDNA (BC105682) for the 50 kDa form of enzyme was cloned into the StrepII C-term vector pCPR0051 (pCEP4-CTStrepII) and the construct verified by sequencing. After transformation into Mach1-T1 cells (Invitrogen) DNA was purified using the Nucleobond PC 10000 EF kit (Macherey–Nagel). Cells were grown in unsupplemented Freestyle 293 expression medium, and incubated in an orbital shaker incubator at 37 °C, 70% humidity, 5% CO_2 and 120 rpm (Ø50 mm). 1 day prior to transfection, HEK293 EBNA cells were re-suspended in fresh media to a cell density of 1.2×10^6 cells/mL and incubated overnight. Approximately 15 min before transfection, cells were harvested, re-suspended to 50 mL in fresh media at a cell density of 20×10^6 cells/mL and incubated until transfection. 2500 μg DNA (50 μg/mL final) and 5 mL of polyethylenimine "MAX" (PEI) (Polysciences) (100 μg/mL solution final) was added directly to the cell suspension. 4 h post transfection, 950 mL of media was added to a final volume of 1 L of cell suspension.

Five days post transfection the supernatants were collected by centrifugation at 6500 rpm for 10 min at 4 °C. After addition of 2 tablets of cOMPLETE protease inhibitor (Roche), the supernatants were stored at −80 °C.

2.2. Purification

CHIT1 was purified using Strep-tag/Strep-Tactin purification system (IBA Technologies). Prior to the purification the supernatant was concentrated by 65% ammonium sulfate precipitation or was supplemented with 2 units of biotin decoy solution, Avadin (IBA) and 10% buffer W (IBA) to adjust to pH 7 (untreated supernatant). Precipitated protein dissolved in 50 mL buffer W (pH 7) or untreated supernatant (240–300 mL) was loaded onto a 5 mL Strep-Tactin superflow H-PR cartridge (IBA, 2-1232-001) at a flow rate of 1–1.5 mL/min using a peristaltic pump. Washing and elution of protein was performed according to the manufacturer's instructions. Fractions containing protein were pooled followed by concentration and exchanged into 50 mM sodium phosphate buffer with Vivaspin (30 000 Da cutoff, GE Healthcare). The protein concentration was determined by the Bradford method using a commercial kit (Bio-Rad) with bovine γ-globulin as a protein reference.

2.3. Examination of enzyme activity towards chitin and cellulose pseudo-substrates

The activity of CHIT1 was determined using the chitin pseudo-substrates; 4-nitrophenyl N-acetyl-β-D-glucosaminide (*p*NP-GlcNAc), 4-nitrophenyl-N,N′-diacetyl-β-D-chitobioside (*p*NP-$(GlcNAc)_2$), 4-nitrophenyl-N,N′,N″-triacetyl-β-D-chitotriose (*p*NP-$(GlcNAc)_3$) and the cellulose pseudo-substrate 4-nitrophenyl-β-D-cellobioside (*p*NP-cellobioside) with a commercial chitinase kit (Sigma). In a standard assay 5 μL enzyme diluted in 50 mM sodium phosphate buffer, pH 6.0, and 45 μL 1 mg/mL substrate dissolved in 50 mM sodium phosphate buffer, pH 6.0 were incubated at 30 °C. Samples (50 μL) were removed after 30 min to 24 h and the reaction was quenched by adding 250 μL of sodium carbonate (0.4 M, pH 11). To limit the conversion of substrates to a maximum of 15%, the enzyme was diluted to 111 μg/mL in the *p*NP-$(GlcNAc)_2$ assay and *p*NP-$(GlcNAc)_3$ assay. Absorbance was measured in a plate reader at 405 nm and corrected for absorption in a control sample with added sodium phosphate buffer, pH 6.0, instead of enzyme. Absorption values were converted into concentrations by the use of a *p*-nitrophenol (Sigma) standard curve.

2.4. Examination of CHIT1 with LacNAc-TMR, LacdiNAc-TMR, Type1-TMR and GlcNAc-TMR substrates

The fluorescently labeled substrates had a tetramethylrhodamine (TMR) tag covalently linked to carbohydrates via a hydrophobic linker (Fig. 1 and Supplementary Fig. 1). Their preparation has been described previously [10,11]. 2 μL of the respective substrates, GlcNAcβ-O-$(CH_2)_8CONH(CH_2)_2NHCO$-TMR (GlcNAc-TMR) (1.5 mM), Gal-β1,4-GlcNAc-O-$(CH_2)_8CONH(CH_2)_2NHCO$-TMR (LacNAc-TMR) (1.9 mM), GalNAc-β1,4-GlcNAc- O-$(CH_2)_8CONH(CH_2)_2$-NHCO-TMR (LacdiNAc-TMR) (1.9 mM), or Gal-β1,3-GlcNAc-O-$(CH_2)_8CONH(CH_2)_2NHCO$-TMR (Type 1-TMR) (1 mM) were incubated with 4 μL of enzyme (CHIT1 68 μg/mL) at room temperature for 30 min to 20 h. Reaction progress was monitored by removing 0.5 μL aliquots for thin-layer chromatography on silica gel plates developed with $CHCl_3$/MeOH/H_2O (65/35/5). TMR-labeled compounds have a bright red color and thus are visible by eye. When appropriate, supernatants from cells lacking expression of CHIT1 were used as a negative control.

Kinetic parameters for LacNAc-TMR and LacdiNAc-TMR were determined by monitoring product formation with 8 different concentrations of substrate ranging from 0.015 to 0.80 mM (LacNAc-