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Characterization of the ERAD process of the L444P mutant glucocerebrosidase variant

Inna Bendikov-Bar [a], Idit Ron [a,1], Mirella Filocamo [b], Mia Horowitz [a,*]

[a] Department of Cell Research and Immunology, Tel Aviv University, Ramat Aviv, 69978, Israel
[b] Laboratorio Diagnosi Pre-Postnatale Malattie Metaboliche, IRCCS G. Gaslini, Genova, Italy

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ABSTRACT

A large number of mutations in the glucocerebrosidase gene (GBA gene), encoding the lysosomal acid hydrolase glucocerebrosidase (GCase), lead to Gaucher disease (GD). The second most prevalent GD causing mutation, carried by 38% of non-Jewish patients, is L444P, resulting from a T to C transition in nucleotide 6092 of the GBA gene. It is a severe mutation that, in homozygosity, leads to neuropathic type 3 GD.
We have previously shown that mutant GCase variants present variable degrees of endoplasmic reticulum (ER) retention and undergo ER associated degradation (ERAD). However, ERAD of the L444P mutant variant of GCase has never been tested. In the current study, we present results indicating that the L444P mutant protein undergoes extensive ERAD. In skin fibroblasts, originated from GD patients homozygous for L444P mutation, the level of GCase is 12%–21% of normal and at least 50% of it is in the ER. The mutant protein undergoes polyubiquitination and proteasome-dependent degradation.
Recently Ambroxol, a known expectorant, was identified as a pharmacological chaperone for mutant GCase. We tested the effect of Ambroxol on the L444P mutant GCase and found that it enhances the removal of the mutant enzyme from the ER. In some cases, this removal leads to a concomitant increase in enzymatic activity.

Introduction

Gaucher disease (GD), resulting mostly from mutations in GBA, a gene encoding the lysosomal acid hydrolase glucocerebrosidase (GCase), has been classically divided into non-neuronopathic type 1 disease (MIM# 230800), infantile acute neuronopathic type 2 disease (MIM# 230900) and juvenile sub-acute neuronopathic type 3 GD (MIM# 231000) [1]. It is evident to date that even type 1 GD patients may develop, later in life, a neuropathy like Parkinson disease [2–5].

GCase is a lysosomal, membrane-associated, glycoprotein. Following its translation on ER-bound polyribosomes and after translocation through the ER membrane, it undergoes N-linked glycosylation on four asparagine residues. The high mannose sugar moieties are further modified while moving through the Golgi apparatus and a 59–63 kDa mature GCase reaches the lysosomes [6].

Newly synthesized proteins are subjected to the ER quality control (ERQC) system that recognizes misfolded proteins and retains them in the ER for refolding by ER chaperone molecules. Proteins that fail to refold are retrotranslocated from the ER to the cytosol, where they are eliminated by the ubiquitin–proteasome system (UPS), through the ER associated degradation (ERAD) process. It is well documented that mutant proteins are also identified as misfolded by the ERQC and are retained in the ER [7]. We have shown that mutant GCase variants present variable levels of ER retention and undergo ERAD in the proteasomes. The degree of ER retention and proteasomal degradation is one of the factors that determine GD severity [8]. However, ERAD of the L444P mutant has not been tested.

The current standard of care for individuals with some lysosomal storage diseases like GD [9,10], Fabry disease [11] and Pompe disease [12] is enzyme replacement therapy (ERT), in which intravenous infused recombinant enzyme is internalized by cells via cell surface receptors and is transported to lysosomes, to break down accumulated substrate. The current ERT for GD is limited to the treatment of non-neurological symptoms, due to the inability of the enzyme to cross the blood–brain barrier [13]. This therapeutic approach is expensive (~$200,000/yr/70 kg) and must be administered intravenously [9]. Lately, a new therapeutic strategy, pharmacological chaperone therapy [14], has been evaluated in a number of clinical trials (information about these studies is available at the USA Clinical Trials website: http://www.clinicaltrials.gov). Pharmacological chaperone therapy uses small molecules, known as pharmacological chaperones (PCs), to stabilize the native conformation of a mutant enzyme as it folds in the ER, thus allowing more functional molecules to form and evade the ERAD pathway. These molecules not only are removed from the ER but are also trafficked to the lysosomes, where the chaperone molecules are displaced, allowing the mutant enzyme to hydrolyze the substrate, if it has residual activity [15]. Pharmacological chaperones are less expensive to manufacture, can be taken orally and, most importantly, cross the blood–brain barrier. A number of competitive inhibitors have been identified as pharmacological

* Corresponding author.
E-mail address: horwitzm@post.tau.ac.il (M. Horowitz).
[1] Present address: Proteologics Ltd., Kiryat Weizmann, Rehovot, 76124, Israel.

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chaperones for GCase [16]. Recently, the iminosugar isofagomine was reported to increase the activity of the L444P mutant GCase variant [17]. Maegawa et al. [18] screened the National Institute of Neurological Diseases (NINDS) library of 1040 drugs, which are Food and Drug Administration (FDA)-approved, and identified Ambroxol, a known expectorant, as a potential pharmacological chaperone for mutant GCase.

In the present study, we show that the L444P mutant GCase undergoes extensive ERAD and that Ambroxol can serve as a pharmacological chaperone for this mutant variant.

Materials and methods

Materials

The following antibodies were used in this study: monoclonal anti-GCase 2E2 (Abnova), generated against a peptide corresponding to amino acids 146-236 of human GCase, rabbit polyclonal anti-GCase, generated against a peptide corresponding to amino acids 517-536 of human GCase (G4171, Sigma), rabbit polyclonal anti-calnexin (Sigma), mouse monoclonal anti-p53 (DO1, kindly provided by Dr D. Lane, Department of Surgery and Molecular Oncology, University of Dundee, Dundee, UK), rabbit anti-Erk (C16 Santa Cruz Biotechnology, Santa Cruz, CA, USA); secondary antibodies cy-3-conjugated goat anti-mouse and cy-2-conjugated goat anti-rabbit, horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit were purchased from Jackson Immuno Research Laboratories, West Grove, PA, USA.

Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinalAc (ALLN) were purchased from Calbiochem (San Diego, CA, USA). Cyclohexamide (CHX) was purchased from Sigma-Aldrich (Israel). Four-methyl-umbelliferyl-glucopyranoside (4-MUG) was purchased from Genzyme Corp. (Boston, MA, USA). Nonident P-40 (NP-40) was purchased from Roche Diagnostics (Mannheim, Germany). Leupeptin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (Israel). Endonuclease-H (endo-H) was purchased from New England Biolabs (Beverly, MA, USA).

Cell lines

Human primary skin fibroblasts, detailed in Table 1, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) (Beit Haemek, Israel) at 37 °C in the presence of 5% CO_2.

Endo-H treatment

Samples of cell lysates, containing 100 μg of total protein, were subjected to an overnight incubation with endo-H, according to the manufacturer's instructions.

Table 1
Genotypes of the different cell lines used in the present study.

Patient #	Genotype	Disease type	GCase level (% of normal)	% endo-H resistant fraction
1	Normal	Normal	100	89.1 ± 4.2
2	L444P/L444P	3	21.1 ± 2.9	49.2 ± 10.3
3	L444P/L444P	3	16.1 ± 2.6	44.7 ± 7.1
4	L444P/recNci	2	12.4 ± 1.4	42.3 ± 6.2
5	L444P/L444P	3	16.5 ± 2.5	49.7 ± 8.2
6	L444P/L444P	3	19.4 ± 4.9	46.1 ± 9.1
7	N370S/N370S	1	61.1 ± 1.45	79.7 ± 4.7

The table represents data on patients whose skin fibroblasts were used in the present study as well as GCase level and its endo-H resistant fraction in each cell line. The results were obtained from experiments presented in Fig. 1.

Proteasome inhibition

Subconfluent human skin fibroblasts, grown on 9 cm plates, were treated with 15 μM ALLN and 25 μM MG-132. Twenty hours later, protein lysates were prepared, and samples, containing the same amount of protein, as determined by the Bradford technique [19] were subjected to Western blot analysis.

SDS–PAGE and Western blotting

Cell monolayers were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C in 500 μl of lysis buffer (10 mM HEPES pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$ and 1% Triton X-100) containing 10 μg/ml aprotinin, 0.1 mM PMSF and 10 μg/ml leupeptin. Lysates were incubated on ice for 30 min and centrifuged at 10,000 × *g* for 15 min at 4 °C. Samples, containing the same amount of protein, were electrophoresed through 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane (Schleicher and Schuell BioScience, Keene, NH, USA). Membranes were blocked with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline (TBS) for 1 h at room temperature (RT) and incubated overnight with the primary antibody. The membranes were then washed three times in 0.1% Tween-20 in TBS and incubated with the appropriate secondary antibody for 1 h at RT. After washing, membranes were reacted with ECL detection reagents (Santa Cruz Biotechnology, Inc.) and analyzed by luminescent image analyzer (Kodak X-OMAT 2000 Processor Kodak, Rochester, NY, USA).

Ubiquitination in tissue culture

Subconfluent skin fibroblasts were treated overnight with 25 μM MG-132, after which stringent immunoprecipitation conditions were applied as previously described [20], with some modifications. The medium was aspirated and the cells were harvested and lysed in 100 μl of denaturing buffer (1% SDS, 50 mM Tris pH 7.4, 140 mM NaCl). The lysates were immediately boiled for 10 min after vigorous vortexing. Then, the samples were cleared by centrifugation at 10,000 × *g* for 10 min and diluted 10 fold into a buffer containing 2% Triton X-100, 50 mM Tris pH 7.4, 140 mM NaCl. After additional centrifugation to remove any insoluble material, immunoprecipitation was carried out with monoclonal anti-GCase antibody. Following three washes (5% sucrose, Tris pH 7.5, 50 mM NaCl, 0.5% NP-40, EDTA 5 mM), the ubiquitin–GCase conjugates were resolved through SDS–PAGE.

Cycloheximide chase experiments

Subconfluent human skin fibroblasts were chased with 100 μg/ml CHX, with or without proteasome inhibitor (25 μM MG-132). At the indicated times, cell lysates were prepared, and samples, containing the same amount of protein, were subjected to SDS–PAGE and Western blotting.

Immunocytochemical analysis and confocal laser scanning microscopy

Subconfluent cells, grown on coverslips, were washed twice with PBS, fixed for 5 min at 4 °C in methanol, followed by 5 min at 4 °C in methanol–acetone (1:1). After washes, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min at RT. Cells were then washed three times with PBS, blocked by incubation with PBS containing 5% bovine serum albumin (BSA) and 20% normal goat serum for 30 min at RT, and then incubated for 1 h with the corresponding primary antibody (1:50 dilution for 2E2 and 1:200 for rabbit anti-calnexin) in 1% BSA in PBS at RT. Following three washes with PBS, cells were immunostained with rabbit-cy-2 or mouse-cy-3 conjugated secondary antibodies (1:200 dilution) in PBS containing 1% BSA for 45 min at RT.