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A nonsense mutation in the LIMP-2 gene associated with progressive myoclonic epilepsy and nephrotic syndrome

Andrea Balreira[1,2,†], Paulo Gaspar[1,†], Daniel Caiola[1], João Chaves[4], Idalina Beirão[5], José Lopes Lima[4], Jorge Eduardo Azevedo[2,3] and Maria Clara Sá Miranda[1,*]

[1]Unidade de Biologia do Lisossoma e do Peroxissoma (UNILIPE) Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto, Portugal, [2]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal, and [3]Biogénese e Funcão de Organelos, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto, Portugal, [4]Serviço de Neurologia and [5]Serviço de Nefrologia, Hospital de S. António, Porto, Portugal

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The main clinical features of two siblings from a consanguineous marriage were progressive myoclonic epilepsy without intellectual impairment and a nephrotic syndrome with a strong accumulation of C1q in capillary loops and mesangium of kidney. The biochemical analysis of one of the patients revealed a normal β-glucocerebrosidase activity in leukocytes, but a severe enzymatic deficiency in cultured skin fibroblasts. This deficiency suggested a defect in the intracellular sorting pathway of this enzyme. The sequence analysis of the gene encoding LIMP-2 (SCARB2), the sorting receptor for β-glucocerebrosidase, confirmed this hypothesis. A homozygous nonsense mutation in codon 178 of SCARB2 was found in the patient, whereas her healthy parents were heterozygous for the mutation. Besides lacking immunodetectable LIMP-2, patient fibroblasts also had decreased amounts of β-glucocerebrosidase, which was mainly located in the endoplasmic reticulum, as assessed by its sensitivity to Endo H. This is the first report of a mutation in the SCARB2 gene associated with a human disease, which, contrary to earlier proposals, shares no features with Charcot–Marie–Tooth disease both at the clinical and neurophysiological levels.

INTRODUCTION

Lysosomal integral membrane protein type 2 (LIMP-2), the product of the SCARB2 gene (1) (MIM* 602257), is a member of the CD36 superfamily of proteins (2,3). LIMP-2 is a ubiquitously expressed 478 amino acid transmembrane protein (4), mainly found in lysosomes and late endosomes (5). Topologically, LIMP-2 has both termini exposed to the cytosol, two transmembrane domains and a 400-residue long luminal domain (6). The targeting and localization of LIMP-2 to late endosomes and lysosomes are mediated by a di-leucine motif present in its C-terminal cytosolic tail (7,8).

Several independent observations suggesting that LIMP-2 is required for the biogenesis and maintenance of the lysosomal/endosomal system have been reported over the past years (9,10). A major advancement, however, was made recently by Reczek *et al.* (11) in a work, showing that LIMP-2 is the mannose-6-phosphate independent trafficking receptor for β-glucocerebrosidase (β-GCase), a lysosomal enzyme that is deficient in most cases of Gaucher disease (GD) (reviewed in 12). The LIMP-2/β-GCase interaction involves amino acid residues 150–167 of LIMP-2 and is much stronger at neutral than at the acidic pH typically found in lysosomes (11). This and other observations led the authors to propose that LIMP-2 binds β-GCase in the endoplasmic reticulum (ER) and escorts the enzyme to the late endosomes/lysosomes, where the acidic pH dissociates the receptor–cargo protein complex (11).

The importance of the LIMP-2-mediated protein sorting pathway is illustrated by the phenotype of LIMP-2 knockout (KO) mice. The absence of this protein in mice causes urinary and neurological alterations (11), associated with impaired vesicular trafficking and distribution of apically

*To whom correspondence should be addressed at: Unidade de Biologia do Lisossoma e do Peroxissoma, IBMC—Instituto de Biologia Molecular e Celular, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal. Tel: +351 226074900; Fax: +351 226074969; Email: mcsamir@ibmc.up.pt
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

expressed proteins (13). An intriguing property of these mice, however, regards the tissue-specific differences observed both at the level of β-GCase activity and accumulation of glucosylceramide, the substrate of β-GCase (11). Indeed, increased levels of glucosylceramide were found in the liver and lungs of these mice, whereas no accumulation was detected in spleen, kidney or brain. Regarding β-GCase activity, a 5-fold decrease was detected in liver extracts of LIMP-2 KO mice (20% of the wild-type level), whereas in brain, this factor was only 1.7. The reason for these differences is presently unknown, although some hypotheses have been forwarded (11).

Given the important role of LIMP-2 in the lysosomal sorting of β-GCase in mice and in cultured human-derived cell lines (11), the possibility that mutations in the SCARB2 gene are associated with disease in humans seems highly likely. In fact, a molecular screening for LIMP-2 mutations in patients with Charcot–Marie–Tooth disease has been initiated (9). However, a disease-associated LIMP-2 mutation has not been described thus far.

In this work, we describe a deficiency in LIMP-2 resulting from a nonsense mutation in the SCARB2 gene. When in a homozygous state, the mutation was associated with progressive myoclonic epilepsy without intellectual impairment and a nephrotic syndrome with strong accumulation of C1q in capillary loops of the kidney.

RESULTS

Clinical phenotype of the patients

Two sisters from a northern Portuguese family (patients 1 and 2, respectively) presenting progressive myoclonic epilepsy and nephrotic syndrome were studied. The patients had an older healthy sister. Their parents were first-degree cousins with a normal neurological examination. Patient 1 started to have myoclonic jerks during active movements of the upper limbs at the age of 15. Symptoms progressed insidiously in intensity, reaching the lower limbs and the trunk later on, developing instability of the gait. When she was 18, she exhibited slowed horizontal saccadic eye movements. A proteinuria of 1 g/24 h was detected at this time. At the age of 20, she was totally dependent, unable to write, to eat alone or to walk, and she had a blurred speech with swallowing difficulties. One year later, she had edemas in both lower limbs and anasarca with bilateral pleural effusions. Nephrotic syndrome, normocytic normochromic anemia and thrombocytopenia were diagnosed. At this time, renal and bone marrow biopsies were performed. The histological analysis of the medullar zone in renal material revealed extensive tubular alterations with isometric vacuolization in distal and collecting tubules and the presence of granular material in cortical tubules without inflammatory infiltration deposits. No 'Gaucher cells' were identified. A marked deposition of C1q and IgM and irregular moderate deposits of C3 in capillary loops and mesangium were observed by immunofluorescence. Bone marrow biopsy was normal. Again, no 'Gaucher cells' were found. She died at the age of 23 with a methicilin-resistant *Staphylococcus aureus* and *Candida albicans* septicemia with multiorgan failure.

Patient 2 developed basically the same type of symptoms as her sister at the age of 17 with only minor differences. When she was 20, she had slowed horizontal saccadic eye movements. At the age of 21, she presented nephrotic syndrome, and 1 year later, she was totally dependent. At the age of 23, she had a normocytic normochromic anemia and hypoalbuminemia, but no thrombocytopenia. Like her sister, she had no hepatosplenomegaly and no intellectual impairment. She died at the age of 26 with a fulminant pneumonia due to an unidentified agent.

β-GCase activity is deficient in skin fibroblasts but not in leukocytes of patient 2

As stated earlier, the two patients described in this study presented a progressive myoclonic epilepsy, a syndrome shared by a group of rare and heterogeneous disorders, such as Niemann–Pick disease type C (MIM #257220) (14,15) and neuronopathic forms of GD (MIM #231000) (reviewed in 16). Owing to their neurological features, namely, the myoclonic jerks and ocular movement abnormalities, they were clinically suspected of Niemann–Pick disease type C, a lysosomal storage disease characterized by lysosomal/late endosomal accumulation of endocytosed unesterified cholesterol (reviewed in 17). However, no cholesterol accumulation, as assessed by filipin staining (18), could be detected in cultured skin fibroblasts from patient 2 (unpublished data), which excluded this disease as the cause of the symptoms.

The existence of an atypical neurological type III GD in these patients was still considered. In order to test this possibility, we determined the activities of β-GCase and several other enzymes in skin fibroblasts, leukocytes and plasma from patient 2, her parents and her healthy sister. As shown in Table 1, patient 2 fibroblasts presented a very low activity of β-GCase (10% of the controls). The measured activity is, in fact, close to the values observed in GD patients' fibroblasts. Strikingly, however, in contrast to GD patients, β-GCase activity in patient 2 leukocytes was normal. The activity of chitothriosidase, an enzyme secreted by activated macrophages and a surrogate marker of GD (19), was also normal in patient 2 plasma. All the other enzymatic activities analyzed were comparable with the control values (Table 1).

Finally, no mutations were found in the β-GCase gene of patient 2 by sequencing of exonic regions and intronic boundaries (unpublished data). Thus, no evidence for the existence of GD in these patients could be found at the biochemical or genetic level.

A homozygous nonsense mutation in the SCARB2 gene in patient 2

Newly synthesized β-GCase is sorted from the ER to the lysosome by LIMP-2 (11). However, for reasons that are still unclear, the absence of LIMP-2 in mice does not result in the same degree of β-GCase deficiency in all tissues (11). Considering the β-GCase activity values described earlier for fibroblasts and leukocytes from patient 2, we decided to analyze the LIMP-2 gene (SCARB2). As shown in Figure 1, sequencing of exonic regions of the LIMP-2 gene revealed a homozygous G-to-A substitution in exon 4 (c.533G>A) in

Table 1. Enzymatic activities of lysosomal enzymes in fibroblasts, leukocytes and plasma

	Enzymes	Patient	Parents		Control ($n = 100$)	GD patients ($n = 25$)
			Father	Mother		
Fibroblasts (nmol/h/mg protein)	β-GCase	33.9	201	171	301.71 ± 89.42	11.2 ± 6.8
	Hex T	7457	7583	7932	5729 ± 1702	7291 ± 3378
	Hex A	233	795	832	542 ± 106	448 ± 218
	β-gal	242	689	836	758 ± 225	496 ± 215
Leukocytes (nmol/h/mg protein)	β-GCase	7	10	12	10.54 ± 2.34	1.1 ± 0.8
	Hex T	1759	1995	2285	1379 ± 442	1978 ± 652
	Hex A	169	394	411	207 ± 56	197 ± 57
	β-gal	178	245	333	219 ± 63	211 ± 73
Plasma (nmol/h/ml)	Chito	26.4	35	34	39 ± 29	11610 ± 9898
	Ac Phosp	529	290	347	229 ± 128	3093 ± 1891
	Hex T	608	858	898	786 ± 222	1900 ± 951
	Hex A	93	60	58	63 ± 15	215 ± 136
	β-gal	9.5	3	3	3 ± 1	13 ± 8

Enzymatic activities of the lysosomal enzymes were determined in samples from patient 2, her parents, controls and GD patients as described in Materials and methods. Values correspond to a representative determination in the case of the patients and parents and to the mean ± SD for controls and GD (n, number of individuals whose enzymatic activity was determined).

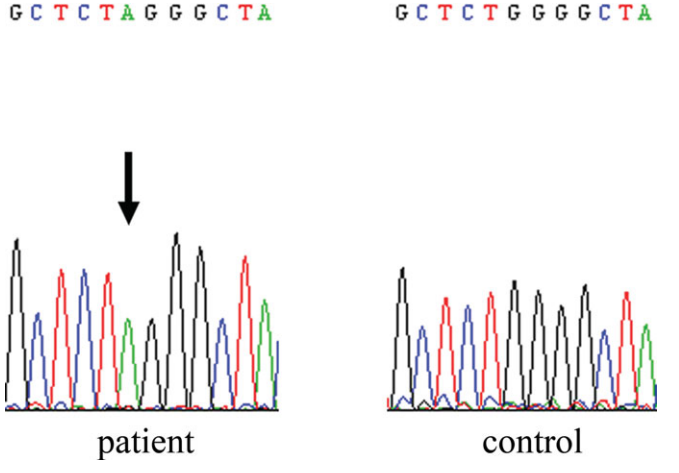

Figure 1. The sequence analysis of the SCARB2 gene revealed homozygosity for c.533G>A mutation. Direct sequencing of exon 4 of the patient and a control revealed the homozygous G-to-A substitution at position 533, resulting in a tryptophan-to-stop codon change. Arrow indicates the G-to-A mutation in the patient.

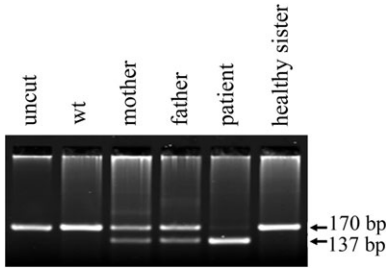

Figure 2. Detection of the c.533G>A mutation by restriction analysis. G-to-A transition at position 533 creates a cleavage site for FspBI. The mutant 170 bp fragment is digested giving two fragments of 137 and 33 bp, whereas wild-type (wt) fragments remain uncleaved. Arrows indicate the 170 and 137 bp fragments.

patient 2. This mutation results in the substitution of a TGG coding for W178 by a premature stop codon. The presence of this mutation in other members of the patient 2 family was studied by restriction enzyme analysis (the G-to-A transition creates a cleavage site for FspBI). As shown in Figure 2, both parents were found to be heterozygous for the c.533G>A mutation. No mutation was found in her healthy sister.

β-GCase in LIMP-2-deficient fibroblasts is decreased and is sensitive to Endo H digestion

As expected from the nonsense mutation found in the SCARB2 gene, no LIMP-2 could be detected in total protein extracts from patient 2 skin fibroblasts (Fig. 3, upper panel), whereas a normal amount of LAMP-2, a lysosomal-associated membrane protein (reviewed in 6), was observed (Fig. 3, middle panel). Interestingly, the steady-state levels of β-GCase in patient 2 fibroblasts were significantly lower than the ones observed in control cells. Moreover, in patient 2 cells, only one protein band was detected with the anti-β-GCase antibody, whereas in control cells, several bands corresponding to different glycosylated forms of β-GCase (20) were observed (Fig. 3, lower panel).

The abnormal glycosylation pattern observed for β-GCase in patient 2 fibroblasts is probably the result of intracellular missorting due to the absence of LIMP-2. In order to corroborate this interpretation, protein extracts from patient 2 and control fibroblasts were subjected to Endo H digestion (21). As shown in Figure 4, the vast majority of β-GCase in normal cells is Endo-H-resistant, implying that most of the protein synthesized in the ER crossed the mid-Golgi and acquired an *N*-glycan complex structure. In contrast, in LIMP-2-deficient fibroblasts, β-GCase is Endo-H-sensitive, suggesting that its high mannose moieties were not yet processed into complex *N*-glycans (Fig. 4, upper panel). Thus, the absence of LIMP-2 in patient 2 fibroblasts leads to a depletion of post-Golgi forms of β-GCase, an observation that is in perfect agreement with the recently described role of LIMP-2 (11).

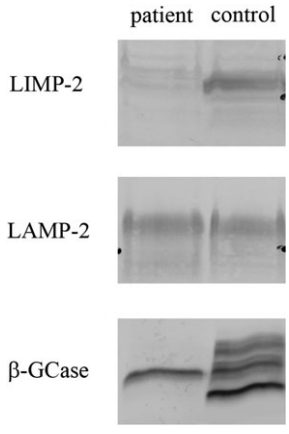

Figure 3. Western blotting analysis of LIMP-2, LAMP-2 and β-GCase in fibroblasts extracts. Fibroblast homogenates with the same amount of protein (80 μg) from the patient and a healthy control were immunoblotted for LIMP-2 (upper panel), LAMP-2 (middle panel) and β-Gcase (lower panel).

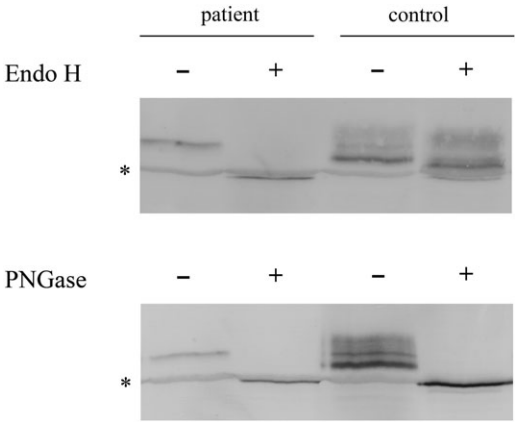

Figure 4. ER retention of β-GCase in LIMP-2-deficient fibroblasts. Fibroblast lysates with the same amount of protein (15 μg) from the patient and a control were treated with Endo-H (upper panel) and PNGase F (lower panel) and immunoblotted for β-GCase. *Unspecific band occasionally detected by the antibody.

DISCUSSION

In this work, we describe for the first time a deficiency in human LIMP-2, the sorting receptor for β-GCase. A biochemical and molecular characterization of one of two sisters presenting identical neurological and renal symptoms revealed the existence of a homozygous nonsense mutation in codon 178 of the LIMP-2 gene. In principle, such a mutation could still allow the synthesis of a truncated protein comprising amino acid residues 1–177 of LIMP-2, a possibility that could not be addressed in this work (the anti-LIMP-2 antibody used here recognizes the C-terminus of the protein). However, even if we assume that such truncated protein is indeed produced, stable and capable of interacting with β-GCase, the fact that it lacks the C-terminal late endosomal/lysosomal targeting information suggests that it would not be functional in sorting its cargo protein(s). As shown by Vega *et al*. (22), the deletion of just 47 amino acid residues from the C-terminus of LIMP-2 renders the truncated protein unable to reach the late endosomal/lysosomal compartments and results in an ER and plasma membrane localization. More significantly, as far as the detection limits of a western blotting analysis allow us to say, no fully matured Endo-H-resistant β-GCase could be found in skin fibroblasts from the patient, an observation strongly suggesting that the majority of the enzyme present in these cells never reaches the Golgi apparatus. Thus, at least in the *ex vivo* studies, the effects of the c.533G>A mutation seem to be quite similar to those observed in mice cells completely lacking LIMP-2 (11).

One intriguing aspect of the data presented here regards the juvenile onset of symptoms in the patients. Apparently, the absence of a fully functional LIMP-2 protein is compatible with a normal life for more than a decade. This is in sharp contrast to the situation observed in GD, in which in the most severe cases, early infantile and even neonatal onsets have been described (23). Therefore, at the phenotypic level, it seems that the lack of the sorting receptor LIMP-2 has less severe consequences than lack of the cargo it sorts, a conclusion also supported by the phenotypes of the LIMP-2 and β-GCase KO mice (11,24). There may be several explanations for this apparent paradox. It is possible that a residual amount of β-GCase reaches the lysosome even in the absence of LIMP-2. Small amounts of β-GCase may be sorted to the lysosome by a direct mechanism, by an as-yet unidentified protein, or indirectly, by endocytosis of secreted β-GCase. Alternatively, it is possible that the relatively large pool of β-GCase detected in pre-Golgi compartments in both normal and LIMP-2-deficient cells (11,21; see also Fig. 4) is more important for the cell lipid homeostasis than presently assumed. Clearly, further work is necessary to clarify these questions.

It is noteworthy that the clinical phenotype of the patients described here does not correlate with the phenotype of LIMP-2 KO mice, despite the neurological and renal involvement observed in both. In LIMP-2 KO mice, neurological alterations translate into deafness and peripheral neuropathy, whereas no such symptoms were observed in the patients. Instead, progressive myoclonic epilepsy without intellectual impairment was the most important neurological feature. At the renal level, LIMP-2 KO mice present an obstruction of the ureteropelvic junction, whereas no urinary tract obstruction was observed in the patients. The patients developed a C1q nephropathy, a relatively rare type of glomerulonephritis with a still unknown pathogenesis mechanism (25).

The absence of a correlation between the LIMP-2 KO mice phenotype and the clinical phenotype of the patients described here has implications for future genetic screening of LIMP-2 deficiencies. For instance, based on the phenotype of the LIMP-2 KO mice, it was previously suggested that some patients with Charcot–Marie–Tooth, a sensorineural peripheral polyneuropathy, might have mutations in the LIMP-2 gene (9). In the light of our findings, this possibility seems now improbable.

While this manuscript was under revision, Berkovic *et al*. (26) described LIMP-2 mutations in three patients with action myoclonus-renal failure syndrome.

MATERIALS AND METHODS

Materials

The monoclonal antibody against human LAMP-2 (H4B4), developed by August and Hildreth, was obtained from the Development Hybridoma Bank under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA. The monoclonal antibody against human β-glucocerebrosidase (8E4) (27) was kindly provided by Professor Aerts, E.C. Slater Institute for Biochemical Research, University of Amsterdam, The Netherlands. The rabbit polyclonal anti-LIMP-2 antibody was kindly provided by Novus Biologicals, Littleton, USA.

4-Methylumbelliferyl (MU)-β-D-glucopyranoside, 4-MU-phosphate-free acid and 4-MU-β-D-galactopyranoside were purchased from Glycosynth; 4-MU-β-*N*-acetylglucosaminide-6-sulfate was purchased from Moscerdam Substrates; 4-MU-*N*-acetyl-β-D-glucosaminide and 4-MU-β-D-*N,N′,N″*-triacetylchitotrioside were purchased from Sigma-Aldrich.

Endo-H and PNGase F were from New England Biolabs. Restriction enzymes were from Fermentas Life Sciences.

Biological samples

Despite the fact that we described two patients clinically, we only had access to biological samples of patient 2. Fibroblasts obtained from skin biopsies were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin–streptomycin, 100 μg/ml kanamycin sulfate and 2.5 μg/ml fungizone (Gibco, Invitrogen). Cells were grown at 37°C in the presence of 5% CO_2. Plasma and leukocytes were separated from whole blood, according to standard methods. DNA was extracted from whole blood with Puregene™ DNA Isolation Kit (Gentra Systems).

Enzymatic activity assays

4-MU-based activity assays were performed for the determination of enzymatic activity of β-GCase, hexosaminidase T (Hex T), hexosaminidase A (Hex A), β-galactosidase (β-Gal), in fibroblasts and leukocytes. Chitotriosidase (Chito), acid phosphatase (Ac Phosp), Hex T, Hex A and β-Gal enzymatic activities were determined in plasma, as described previously (28–31).

Molecular analysis

Regions of the SCARB2 gene were amplified and sequenced using the following primers: SCARB2-exon 3-F: 5′-ATGTTTTTGAACGAGACCAATCTGT-3′; SCARB2-exon 3-B: 5′-TCCTTTCTTCCAAAATCCAACTTA-3′; SCARB2-exon 4-F: 5′-TGCTGTTCTTAATATACCTCTGCTCC-3′; SCARB2-exon 4-B: 5′-GGATAAGGGACAAGATTTCATCTTT-3′; SCARB2-exon 9-F: 5′-TTTAATAAATCCTAATGTTTCCTTTTCACTTC-3′; SCARB2-exon 9-B: 5′-AACTGCTGTCCCCTCCATAGAA-3′; SCARB2-exon 12-F: 5′-GAACATCTAACTTGTTTTCTCT-3′ and SCARB2-exon 12-B: 5′-ATGATTTTATAAAGCTTAATGG-3′.

SDS–PAGE and western blotting

Fibroblast monolayers at 90% confluence were harvested, washed three times in phosphate-buffered saline and sonicated at 4°C in SEM buffer (0.25 M sacarose, 20 mM MOPS-KOH, pH 7.2, 1 mM EDTA-NaOH, pH 7.2), supplemented with 2 μg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide. Lysates were centrifuged at 1000*g* for 10 min at 4°C. Proteins were quantified by the Bradford technique (32). Samples containing the same amount of protein were subjected to SDS–PAGE and electroblotted onto a nitrocellulose membrane (Schleicher&Schuell). Membranes were blocked with 5% skimmed milk and 0.1% Tween-20 in Tris-buffered saline (TBS) for 1 h at room temperature and incubated overnight with the primary antibodies at 4°C. Membranes were then washed twice with 0.1% Tween-20 in TBS and incubated with the appropriate secondary antibodies for 1 h at room temperature. After washing, detection was performed using alkaline-phosphatase substrates.

Endo H and PNGase treatment

Samples of fibroblast lysates, containing 15 μg of total protein, were treated overnight with Endo-H or PNGase F, according to the manufacturer's instruction (New England BioLabs).

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Conflict of Interest statement. None declared.

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