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Authors
Tokhtaeva, Elmira
Mareninova, Olga A
Gukovskaya, Anna S
et al.

Publication Date
2017

DOI
10.1007/978-1-4939-6934-0_3

Peer reviewed

HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2019 July 29.

Published in final edited form as:

Methods Mol Biol. 2017 ; 1594: 35–42. doi:10.1007/978-1-4939-6934-0_3.

Analysis of *N*- and *O*-Glycosylation of Lysosomal Glycoproteins

Elmira Tokhtaeva, **Olga A. Mareninova**, **Anna S. Gukovskaya**, **Olga Vagin**

Abstract

The vast majority of lysosomal proteins are heavily glycosylated. The present protocol describes the method of analyzing *N*- and *O*-linked glycans in lysosomal proteins of interest. The method is based on using deglycosylating enzymes, endoglycosidases, and exoglycosidases. Endoglycosidases catalyze the cleavage of an internal bond in an oligosaccharide, while exoglycosidases remove terminal carbohydrates from glycans. Different types of carbohydrate residues or chains can be removed by specific glycosidases. Removing oligosaccharides with glycosidases increases the electrophoretic mobility of a protein. This increase in mobility depends on the size and number of removed carbohydrate chains. Therefore, the treatment of lysosomal proteins with specific glycosidases followed by a western blot analysis of a protein of interest provides a way to determine which types of glycans are present in the protein by comparing the gel mobility before and after treatment.

Keywords

Glycosidases; Glycosylation; Lysosomes; *N*-glycans; *O*-glycans

1 Introduction

Lysosomes are composed of soluble and transmembrane proteins that are targeted to lysosomes in a signal-dependent manner [1]. More than 60 soluble and more than 100 transmembrane lysosomal proteins have been identified [2, 3], but recent proteomic studies suggest that this list should be largely expanded [4, 5]. The most abundant transmembrane proteins of the lysosomal membrane are the lysosomal-associated membrane proteins LAMP-1 and LAMP-2. LAMPs play critical role in the function of lysosomes, and deficiency of LAMP proteins is associated with disease development [3]. Lysosomal proteins have to withstand intralysosomal acidic pH < 5 and proteolytic activity of multiple acidic hydrolases and hence are usually heavily glycosylated [2, 4, 5].

Both soluble and transmembrane lysosomal proteins are synthesized in the rough ER, where they are modified by *N*-linked glycans that are added to the asparagine of NXS or NXT (where X is any amino acid except proline) motifs in the nascent protein co-translationally. Immediately after coupling, *N*-glycans are modified by the ER-resident enzymes. The modification of *N*-glycans continues during the transit of glycoproteins through the Golgi complex until they reach the trans-Golgi network (TGN) (Fig. 1a). Many lysosomal proteins, including LAMP-1 and LAMP-2, are additionally modified by *O*-linked glycans that are

added to selected serine or threonine residues [7]. Sorting of lysosomal transmembrane proteins depends on the presence of sorting signals in their cytosolic domains. These signals interact with clathrin adaptors that facilitate transport of these proteins to lysosomes either directly or via surface delivery followed by clathrin-dependent endocytosis [1, 8] (Fig. 1a). The majority of soluble acid hydrolases are modified with mannose 6-phosphate residues in the Golgi, allowing their recognition by mannose 6-phosphate receptors that facilitate transport of these proteins from the TGN to the endosomal/lysosomal compartments [9].

This protocol describes methods of analysis of *N*-and *O*-glycans attached to lysosomal proteins using deglycosylating enzymes. Treatment with glycosidases increases the electrophoretic mobility of a protein. This increase is more or less profound depending on the size and number of removed carbohydrate residues or chains. Therefore, following the gel migration before and after treatment with glycosidases provides a convenient way to understand which types of glycans are present in a protein of interest.

Deglycosylating enzymes are classified as endoglycosidases and exoglycosidases. Endoglycosidases are enzymes that catalyze the cleavage of an internal glycoside bond in an oligosaccharide. Exoglycosidases are enzymes that remove terminal carbohydrates from the non-reducing end of a glycan, but do not cleave internal bonds between carbohydrates. Endoglycosidase H (Endo H) cleaves high-mannose and some hybrid oligosaccharide chains from *N*-linked glycoproteins [10]. The most common endoglycosidase, Peptide-*N*-Glycosidase F (PNGase F), catalyzes the deglycosylation of most *N*-linked glycoproteins and cleaves high-mannose, hybrid and complex oligosaccharide chains [10] (Fig. 1b). Exoglycosidase α-Mannosidase cleaves terminal mannose residues [11] and hence can be used as a tool to determine the presence of high-mannose oligosaccharide chains in *N*-linked glycans (Fig. 1b). Neuraminidase is an exoglycosidase that cleaves terminal *N*-acetyl neuraminic acid (sialic acid) residues from glycoproteins. *O*-glycosidase is an endoglycosidase that cleaves Core 1 and Core 3 *O*-linked glycans from glycoproteins, but this cleavage is effective only after removing terminal sialic residues by a neuraminidase. The treatment with these two enzymes removes some but not all *O*-glycans from glycoproteins [12]. Unfortunately, the enzymes that remove all *O*-linked glycans from glycoproteins are not available. Therefore, the analysis of glycoproteins using *O*-glycosidase and neuraminidase cannot detect all *O*-glycans linked to a protein of interest. In other words, the increase in the electrophoretic mobility of a protein after treatment with these two glycosidases is indicative of the presence of *O*-glycans. However, a lack of change in the electrophoretic mobility cannot be interpreted as the complete absence of *O*-glycans.

2 Materials

1. **Enzymes:** Peptide-*N*-Glycosidase F (PNGase F) from *Flavobacterium meningosepticum* (New England BioLabs), endoglycosidase H (Endo H) from *Streptomyces plicatus* (Glyco-Prozyme Inc.), Jack bean α-Mannosidase (Sigma-Aldrich) and *O*-glycosidase & Neuraminidase Bundle (New England BioLabs).
2. Denaturing Solution (10×): 5% SDS (sodium dodecyl sulfate), 400 mM DTT (dithiothreitol) (*see* Note 1).

3. NP40 (10×): 10% NP40 (*see* Note 2).
4. Reaction Buffer for PNGase F and for *O*-glycosidase & Neuraminidase Bundle (10×): 500 mM Sodium Phosphate buffer pH 7.5 (*see* Note 1).
5. Reaction Buffer for Endo H (10×): 500 mM sodium acetate buffer pH 6.0 (*see* Note 1).
6. Reaction Buffer for a-Mannosidase (10×): 250 mM sodium acetate buffer pH 5.0, 10% Triton X-100.
7. SDS-PAGE sample buffer (2×): 0.1 M Tris–HCl buffer pH 6.8, 4% SDS, 0.05% bromophenol blue, 20% glycerol, 1% β-mercaptoethanol.
8. Primary antibodies for western blot analysis: rabbit polyclonal antibody against the C-terminal region of rat and mouse LAMP-1 (ab24170; Abcam, Cambridge, MA), rat monoclonal antibody against full length mouse LAMP-1 (1D4B; Developmental Studies Hybridoma Bank), University of Iowa, Iowa City, IA), and rat monoclonal antibody against full length mouse LAMP-2 (GL2A7; Developmental Studies Hybridoma Bank).
9. Secondary antibodies for western blot analysis: horseradish peroxidase linked goat anti-rat, horseradish peroxidase linked goat anti-rabbit.

3 Methods

3.1 Denaturing Proteins (See Note 3)

1. Combine 1–9 μl of microsome membranes containing 2–30 of protein (*see* Note 4), 1 μl 10× of Denaturing Solution and H_2O (if necessary) to make a 10 μl total reaction volume.
2. Denature proteins by heating the reaction mixture at 100 °C for 10 min.
3. Chill denatured proteins on ice and spin for 10 s.

3.2 Deglycosylation Using PNGase F

1. Combine 10 μl of chilled denatured proteins from Subheading 3.1, step 3 (*see* Note 5) with 2 μl of 10× Reaction Buffer for PNGase F, 2 μl 10× NP40 (*see* Note

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1. Most vendors of deglycosylating enzymes provide 10× denaturing and reaction buffers.
2. PNGase F is inhibited by SDS, thus enough NP40 needs to be added after denaturation to prevent PNGase F inactivation.
3. Deglycosylation is usually more effective under denaturing conditions (using SDS and heat). However, some glycoproteins aggregate as a result of boiling. Optimal denaturing conditions for each glycoprotein should be determined experimentally. For several glycoproteins, including LAMP-1, we found the best results after incubating in the denaturing buffer at 80 °C for 5 min. If necessary, deglycosylation can be performed under native conditions without the addition of detergents. However, longer incubation time and/or more enzyme may be required to deglycosylate native glycoproteins. Also, when deglycosylation of membrane proteins is performed, the presence of non-ionic detergents is required to keep these proteins in solution. The control reaction in denaturing conditions is recommended to confirm that deglycosylation in native conditions is efficient.
4. Instead of membrane preparations, purified glycoproteins, immunoprecipitated protein complexes, or proteins of total cell lysates can be used. When total lysates are used, precautions should be taken to inhibit cytoplasmic and lysosomal proteases during the deglycosylation reaction. The following protease inhibitors can be added to the reaction mixture: aprotinin (10 μg/ml), benzamidine (1 mM), pepstatin (10 μg/ml), leupeptin (1 μM), EGTA (1 mM), EDTA (1 mM), PMSF (phenyl-methylsulfonyl fluoride; 1 mM), or Pefabloc SC (1 mM). A 1000× concentrated stock of each inhibitor can be made in water except for pepstatin and PMSF, which should be dissolved in methanol or DMSO (dimethyl sulfoxide). Note that PMSF has the ability to modify basic residues on glycoprotein substrates.

2) and 5 μl H_2O and 1 μl PNGase F (*see* Note 6) to make a total reaction volume of 20 μl.

2. Incubate reaction at 37 °C for 1 h.

3.3 Deglycosylation Using Endo H

1. Combine 10 μl of chilled denatured proteins from Subheading 3.1, step 3 with 2 μl of 10× Reaction Buffer for Endo H, 1–5μl Endo H and H_2O to make a total reaction volume of 20 μl (*see* Note 7).

2. Incubate reaction at 37 °C for 3 h.

3.4 Deglycosylation Using α-Mannosidase

1. Combine 10 μl of chilled denatured proteins from Subheading 3.1, step 3 with 2 μl of 10× Reaction Buffer for Jack been α-mannosidase, 1–3 μl Jack been α-mannosidase and H_2O to make a total reaction volume of 20 μl (*see* Note 8).

2. Incubate reaction at 37 °C for 3 h.

3.5 Deglycosylation Using O-Glycosidase &Neuraminidase Bundle

1. Combine 10 μl of chilled denatured proteins from Subheading 3.1, step 3 with 2 μl of 10× Reaction Buffer for PNGase F, 2 μl 10× NP40 (*see* Note 2), H_2O, 2 μl Neuraminidase, and 1–5 μl *O*-Glycosidase (*see* Note 9) to make a total reaction volume of 20 μl.

2. Incubate reaction at 37 °C for 1 h.

3.6 Western Blot Analysis of Deglycosylated Proteins

1. After completing the incubation with enzymes, place the reaction mixture on ice and combine it with equal volume of 2× SDS-PAGE sample buffer.

2. Separate proteins by SDS-PAGE.

3. Analyze the lysosomal glycoproteins by western blot using appropriate antibodies (*see* Note 10).

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5. Some lysis buffers contain high NaCl concentrations that can inhibit PNFase F. It is recommended to keep the total molarity of the reaction below 200 mM, and the total SDS concentration not higher than 1%.

6. When purchasing PNGase F, check if it contains glycerol. Since glycerol can inhibit the enzyme activity, limit the amount of the enzyme in the total reaction mixture so that the final glycerol concentration is equal to (or less than) 5%.

7. Endo H enzymatic activity is not affected by SDS, thus there is no need to add NP40.

8. To prevent inactivation of the enzyme by SDS, 1% Triton X-100 is present in the 1× Reaction Buffer for Jack bean α-Mannosidase.

9. *O*-glycosidase removes *O*-linked disaccharides from serine and threonine residues at the attachment site, while Neuraminidase is an exoglycosidase that cleaves *N*-acetyl neuraminic acid (sialic acid) residues from glycoproteins. The former needs trimming of glycoproteins by the latter for its enzymatic action. The treatment with the two enzymes removes some but not all *O*-glycans from glycoproteins [12]. Unfortunately, there is no endoglycosidase that can completely remove all *O*-linked glycans. A more efficient removal of *O*-glycans can be achieved by adding several different exoglycosidases to *O*-glycosidase. A complete removal of *O*-glycans can be achieved by using chemical methods, but they often result in the degradation of the protein.

10. The selection of the right antibody to determine the efficiency of deglycosylation by western blot analysis is crucial. Many glycoprotein-reacting antibodies have different affinities to glycosylated and deglycosylated forms of a protein. An example is provided in Fig. 2. Microsome membranes isolated from a mouse pancreas were analyzed by western blot analysis using two different anti-LAMP-1 antibodies. Both antibodies detect a single wide band at 100 kDa corresponding to the predicted molecular mass of the fully glycosylated LAMP-1 (Fig. 2a, b, left lane). The treatment with Endo H or Jack bean α-mannosidase hardly affected the electrophoretic mobility of the band, while the treatment with PNGase F resulted in a significant increase in mobility, producing a

Acknowledgement

This study was funded, fully or in part, by National Institute of Health grants R01HL113350 (O.V.), P01DK098108 (to A.S.G), and R01AA19730 (to O.A.M.)

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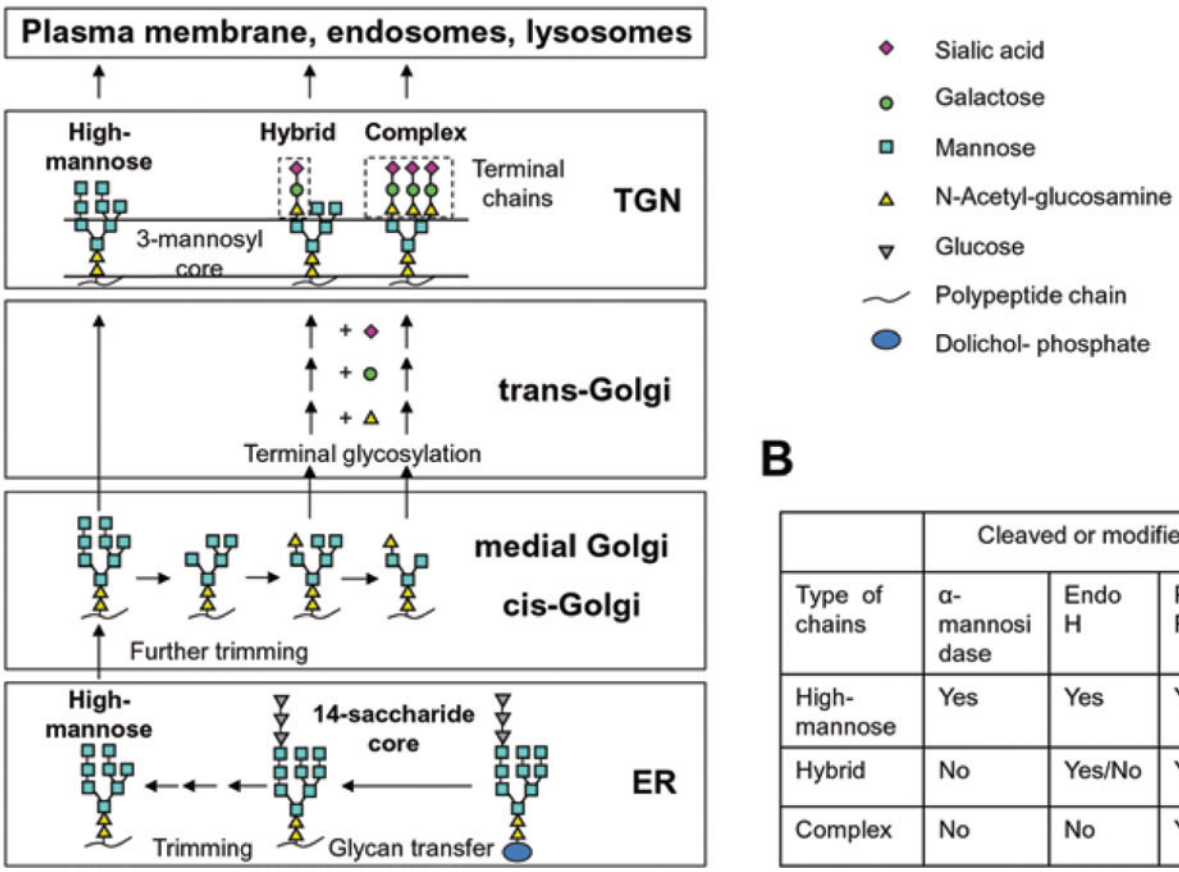
band at 50 kDa as detected with the antibody reacting with the cytoplasmic C-terminal domain of LAMP-1. These data indicate that LAMP-1 has predominantly complex-type *N*-glycans that are resistant to cleavage by Endo H or α-mannosidase (Fig. 1b). Surprisingly, the band corresponding to the deglycosylated LAMP-1 was not detected by the antibody raised against the full-length LAMP-1 (Fig. 2b), suggesting that this antibody reacts exclusively with the glycosylated form of LAMP-1. Similarly, the antibody raised against the full-length LAMP-2 reacts with the fully glycosylated protein (Fig. 2c, left lane), but not with the deglycosylated product (Fig. 2c, right lane). By contrast, many other antibodies react better with the deglycosylated or less glycosylated forms of proteins than with their fully glycosylated forms. Therefore, it is important to confirm that the antibody of your choice equally reacts with glycosylated and deglycosylated forms of the glycoprotein of interest. Usually, it is safer to use the antibodies that react with cytoplasmic epitopes that are not affected by *N*- or mucin-type *O*-glycosylation.

A



B

	Cleaved or modified by		
Type of chains	α-mannosidase	Endo H	PNGase F
High-mannose	Yes	Yes	Yes
Hybrid	No	Yes/No	Yes
Complex	No	No	Yes

Fig. 1.

Analysis of *N*-glycosylated proteins using glycosidases. (**a**) Cartoon showing *N*-glycosylation and trafficking pathways of lysosomal membrane proteins. (**b**) Specificity of glycosidases to different type of oligosaccharide residues/chains in *N*-glycans. Modified from [6]

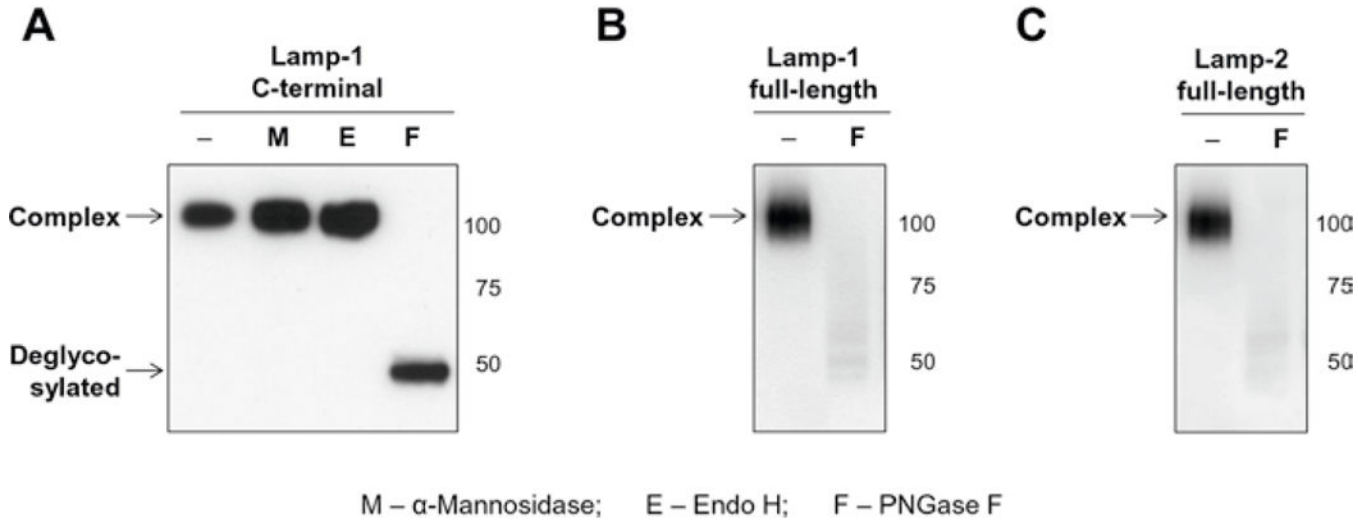


Fig. 2.

Deglycosylation of LAMP-1 and LAMP-2 as analyzed by different antibodies. Microsomal membrane proteins were treated with glycosidases as indicated and analyzed using antibodies against the (**a**) C-terminus of LAMP-1, (**b**) full-length LAMP-1 or (**c**) full-length LAMP-2. Modified from [13]