Published in final edited form as:

Methods Mol Biol. 2013 ; 980: 331–340. doi:10.1007/978-1-62703-287-2_19.

Analysis of Tumor-Associated Mucin Glycotopes by Western Transfer Methods

Vinayaga S. Gnanapragassam, Maneesh Jain, and Surinder K. Batra

Abstract

In mucins, glycosylation is complex and the most predominant posttranslational modification. Since mucins exhibit differential glycosylation pattern under physiological and pathological conditions, analysis of mucin glycans is important for understanding their specific functions during pathological conditions like cancer. Given the complexity of mucin glycans, several sophisticated analytical tools such as HPLC, mass spectrometry, and lectin sandwich assays are employed for glyco-analysis of mucins. However the specialized expertise and instrumentation required for such analysis are beyond the reach of an average cancer biology laboratory. We described in this chapter the utility of the simple electrophoresis/immunoblotting method to examine the mucin glycan epitopes, using specific antibodies and lectins.

Keywords

Mucin; Mucin glycans; Mucin-associated carbohydrate glycans; Tumor-associated carbohydrate antigen; Mucin immunoprecipitation; Western blot/Western transfer; Lectins

1. Introduction

Mucins are high molecular weight glycoproteins, classified as membrane-associated and secreted forms. They are uniquely heavily *O*-glycosylated and sparsely *N*-glycosylated (1). The role of mucins is well established in various stages of oncogenesis from neoplastic transformation to metastasis of epithelial cancers including pancreatic, ovarian, lung, and breast cancer (2). The functions of mucins are highly influenced by their associated glycans, as evidenced from our own observations and studies published by others (3–8, 11, 12). Given the functional role of mucins and associated glycans in cancer and their potential as biomarkers, there is growing interest in developing simpler methods of mucin glyco-analysis that can be performed in a simple laboratory setting. While SDS–polyacrylamide gel electrophoresis (SDS–PAGE) is the most commonly used gel-based separation technique in the protein/glycoprotein analysis (8), it is inefficient in resolving mucins due to their large molecular size. SDS–agarose gel electrophoresis (SDS–AGE)-based methods have been successfully employed for the resolution of large mucins. The choice of method to transfer proteins to PVDF/nitrocellulose membrane is dictated by the type of gel used for resolving the mucins (9). While the SDS–PAGE-resolved proteins can be easily transferred to the PVDF membrane using electrophoretic methods (10), capillary transfer method is usually employed for transferring proteins from agarose gel to PVDF/nitrocellulose membrane. We have successfully employed SDS–agarose gel for the resolution of very high molecular weight mucins like MUC1, MUC4, and MUC16. We have also used this method for the analysis of mucin-associated glycans that involves immunoprecipitation of mucins by anti-mucin antibodies and immunoblotting with anti-glycan epitope antibodies.

2. Materials

All the reagents must be prepared in the ultrapure water unless otherwise specified. We prepare stock solutions for some of the listed reagents, while others are prepared fresh. For cell culture and lysate preparation, routine standard methods and reagents are used. We have observed that cell confluency often impacts mucin levels in the cells, and hence we advise readers to always seed and harvest cells at a consistent cell density.

2.1. Cell Lysis Buffer

1. *2× cell lysis buffer–RIPA (radioimmunoprecipitation assay) buffer*: 7.9 g of Tris base (50 mM), 4.5 g of NaCl (150 mM) added to 375 ml of ultrapure water and mixed well using magnetic stirrer, until dissolved completely. 50 ml of NP-40 (10% stock), 12.5 ml of sodium deoxycholate (10% stock), and 5 ml of EDTA (100 mM stock) are added, the pH is adjusted to 7.4, and the final volume is adjusted to 500 ml. The lysis buffer is filter sterilized and stored at 4°C (see Note 1).
2. *Protease inhibitors*: complete protease inhibitor (Roche), sodium orthovanadate, sodium fluoride, and PMSF are prepared at 200 mM stock at stored at –20°C. The inhibitors are added freshly to the RIPA buffer to a final concentration of 1mM (see Note 2).

2.2. Immunoprecipitation

Protein A and G agarose resins is purchased from GE Amersham Biosciences.

Antibodies for MUC4 and glycan epitopes and IgG control Abs were produced at UNMC Antibody Core Facility. Antibodies to other mucins and glycotopes can be obtained from a commercial source. Biotinylated lectins, streptavidin–HRP, and streptavidin–FITC are purchased from Vector Labs.

2.3. SDS–Polyacrylamide and SDS–Agarose

6× sodium dodecyl sulfate (SDS) sample buffer: 7 ml of 4× Tris–Cl/SDS pH 6.8, 3.0 ml glycerol, 1 g SDS, 0.93 g DTT or 5% BME, and 12 mg of bromophenol blue top up to 10 ml by ultrapure water aliquoted and frozen at –20°C.

1. 2× SDS sample buffer is prepared accordingly with 1 ml of 0.5 M Tris–HCl pH 6.8, 1 ml of 10% SDS, 1 ml of glycerol, 0.1 ml β-mercaptoethanol, and 250 μl of 0.025–0.05% bromophenol blue. Adjust the final volume to 10 ml using ultrapure water. We prepare different buffers for the SDS–polyacrylamide and SDS–agarose gel analysis of mucins and mucin glycans.
2. For the SDS–polyacrylamide analysis, we prepare individual reagents as a stock, based on the need we make fresh working solution. 30% acrylamide solution is purchased from the vendor; 1.5 M Tris pH 6.8 and 8.8 was prepared and stored at 4°C (see Note 3).

¹Cell lysate are obtained using 1× RIPA. To 2.5 ml of 2× RIPA we add 200 μl of complete protease inhibitor cocktail to achieve a final concentration of 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF. The final volume is adjusted to 5 ml of with ultrapure water and kept on ice. Protease inhibitors are prepared as individual stocks, with complete protease inhibitor (25×), sodium orthovanadate, sodium fluoride, and PMSF as 200 mM concentration.

²To prevent the loss of activity, complete protease inhibitor cocktail and sodium orthovanadate should be stored frozen at –20°C in 200 μl aliquots.

³The cell lines are subjected to the trypsinization; trypsin quantity varies based on the surface area of the tissue culture flask. Three and five milliliters of trypsin were employed to the T25 and T75 flask. Tissue lysates are prepared by the freeze–thawing in liquid nitrogen freezing and mechanical disruption by means of pestle and mortar; the frozen tissue is ground into a fine powder and immediately transferred to a tube containing lysis buffer.

3. 10% of SDS and 10% ammonium persulfate (APS) solutions were prepared and stored appropriately. We use commercially available TEMED at appropriate volume.
4. 4× Tris–HCl pH 8.8 (1.5 M Tris–Cl and 0.4% SDS) is prepared for the SDS–acrylamide gel. For 500 ml of 4× buffer, we add 91 g of Tris base to 300 ml ultrapure water. The pH is adjusted using 1 N HCl and the volume is adjusted to 500 ml. The buffer is filtered using 0.2 micron filter, and 2 g of SDS is added (see Note 4).
5. Tris–glycine running buffer: for preparing 2 l of running buffer, 6 g Tris (25 mM), 28.8 g glycine (115 mM, pH 8.3), and 2 g SDS (0.1% SDS) are added in deionized water.

2.4. Western Blot

PVDF membrane was purchased from Millipore.

1. *Transfer buffer*: for 2 l, 6 g Tris (25 mM), 28.8 g glycine (115 mM, pH 8.3), and 0.5 g SDS (0.025% SDS) in 1,600 ml of ultrapure water and 400 ml of methanol is added (20% final conc).
2. *10× PBS*: 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄ 7H₂O, and 2 g KH₂PO₄, for the working solution 100 ml is added to 900 ml of ultrapure water and mixed well. This will give us 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ 7H₂O, 2 g KH₂PO₄, and 1.4 mM KH₂PO₄.
3. *10× TBS*: 24.08 g Tris–HCl, 80.06 g NaCl, are added to 800 ml of ultrapure water, and adjust the pH to 7.6 with HCl and make up the final volume to 1,000 ml. Working solution can be obtained by diluting the stock ten times with ultrapure water.
4. 1 ml of Tween 20 is added to 1× PBS/TBS to obtain 0.1% Tween 20 containing 1× TBST/PBST.
5. 5% Nonfat milk powder in PBS or 2% BSA in TBS is used for blocking.
6. Vertical Mini-PROTEAN (Bio-Rad) gel apparatus is used for the SDS–PAGE, while horizontal gel electrophoresis apparatus is used for the SDS–agarose gel electrophoresis.
7. Clean forceps for handling the PVDF membranes and other accessories as needed.

3. Methods

3.1. Procuring the Cell Lines and Tissues and Obtaining Respective Lysates

The overall scheme for mucin glycan analysis is outlined in Fig. 1.

1. Cell lines were received from the ATCC and both the normal and cancerous tissues from the tissue bank. We choose two different cell lines which are confirmed for the expression of MUC4 at mRNA, and protein level for this study. Normal colon and pancreatic cancer tissue were obtained from the tissue bank.

⁴Protein A + G agarose mixture is equilibrated with the binding buffer (RIPA). The beads are pipetted using a cut micropipette tip into a 1.5 ml tube containing RIPA. The protein A + G agarose resin is centrifuged at $0.4 \times g$ for 2 min at 4°C, and the supernatant is removed. 1 ml RIPA were added and incubated with gentle rotation for 5 min and this step is repeated three times. In the addition of fresh binding buffer, gentle rotation for 30 min will ensure the proper equilibration of the beads.

2. Cell lines were cultured according to the ATCC recommendation till the monolayer is 60–80% confluent. We prefer to culture the cells in 10 cm petri dishes; the cells were cultured in Dulbecco's Modified Eagles Medium containing 10% FBS, 1% penicillin/streptomycin. Prior to trypsinization, the media is aspirated, and the cells are washed twice with PBS (see Note 4). 5 ml of trypsin is added to the cell monolayer and incubated at 37°C for 2–3 min. Trypsin is neutralized by the addition of equal volume of 10% FBS containing DMEM, and cells are centrifuged at $2,500 \times g$ for 2 min. The pellet is suspended in 5 ml of DMEM medium and mixed five times using serological pipet to obtain homogenous cell suspension. The cells are counted using automated cell counter and seeded at 2×10^6 cells/petri dish.
3. When the cells reach a desired cell density, media were aspirated, and the cells are washed with DPBS twice and kept on the ice. 0.5–1.0 ml of RIPA is added and the plates are gently swirled and kept in ice for 1–2 min. Using sterile disposable cell scraper, the cell lysate was collected and transferred in a sterile 1 ml microcentrifuge tube.
4. We carry out mechanical disruption using 1 ml tuberculin syringe (slip tip), with 25 G 7/8 needle, by passaging 15–20 times on ice. The cell lysate obtained was stored at -80°C at least for 3 h, followed by thawing on ice. The lysate is centrifuged at 16.1 RCF for 30 min at 4°C . The supernatant obtained is transferred to a fresh microcentrifuge tube. Protein estimation of the cell lysates is done by Lowry-based Bio-Rad DC Protein Assay, and the samples are stored at -80°C until used.

3.2. Immunoprecipitation of MUC4 by Anti-MUC4/Anti-glycotope Antibodies

1. For immunoprecipitation procedure, we routinely use 1 mg of total protein; however if we analyze other mucins based on the expression (semiquantitative measurement by Western blot), more protein can be used. For routine purpose, we employ 1 $\mu\text{g}/\mu\text{l}$ of total cell lysate to final volume of 1,000 μl of RIPA. For each sample and antibody, respective isotype control should be used.
2. 5 μg of anti-MUC4 (or other anti-mucin) antibody and control antibody is added separately to the diluted cell lysates. The antigen–antibody mixture is kept at gentle rotation at 4°C overnight, which enables the formation of antigen–antibody complex.
3. The protein A + G agarose is used after equilibration with the RIPA buffer. For single assay 30 μl of the protein A + G agarose slurry is used. The equilibration is carried out by adding 1 ml of RIPA and allowed to mix gently at 4°C for 3×5 min and centrifuge at 0.4 RCF for 3 min, followed by addition of fresh 1 ml RIPA. Final equilibration is done by addition of equal volume of RIPA, followed by gentle rotation at 4°C for 30 min (see Note 4).
4. To the each sample tube, 30 μl of resin (protein A + G agarose) is added and kept for gentle rotation for 4 h at 4°C . Centrifugation is carried out at 0.4 RCF for 3 min; the supernatant is aspirated and stored separately. Beads are washed by adding 1 ml RIPA buffer followed by gentle rotation for 5 min. Then centrifugation is carried out as performed for equilibration of resin, and the supernatant is aspirated after the final washing step.
5. Equal volume of $2\times$ sample buffer is added to the agarose beads that contain antigen–antibody complex, and the samples are heated for 4 min at 95°C , cooled on ice, and stored at -20°C until used.

3.3. SDS–Agarose (2%) Gel Electrophoresis

1. 6 g of agarose is added to 225 ml of ultrapure water in a glass bottle. Microwave for 5 min and gently swirl every 30 s (see Note 5).
2. 75 ml of 4× SDS running buffer, pH 8.8, is added to the agarose solution, and the mixture is further heated for 2 min with intermediate swirling. Allow the solution to cool to about 50°C and gently pour into the gel tray without creating air bubbles. Place the combs and allow the agarose to solidify for at least 30 min.
3. The gel tray with slab is placed in the horizontal electrophoresis apparatus, and 2.7 l of running buffer is added to the tank. Immunoprecipitated sample should be heated for 15 s at 95°C and kept in ice for few minutes, followed by centrifugation at $0.4 \times g$, for 3 min.
4. 15 µl of the supernatant is added from the each sample along with the IgG control and the whole cell lysate in separate wells. Electrophoresis should be carried out at 100 V for 6 h at room temperature.

3.4. Nonelectrophoretic Capillary-Mediated Transfer

1. Cut a Whatman filter paper and the PVDF membrane to the approximated dimensions of the slab gel before the electrophoresis is completed. Activate the PVDF membrane with 100% methanol for 0.5 min and equilibrate the membrane and Whatman paper with the transfer buffer for 15 min.
2. As soon as electrophoresis is completed, the gel should be removed and kept in the inside of the lid. The apparatus was washed under running warm water and prepared for the transfer.
3. The two buffer tanks of the horizontal gel apparatus are filled with 600 ml of transfer buffer, and the empty gel tray is placed in an inverted position between the two compartments. Precut Whatman filter paper soaked in the buffer re-placed over the inverted tray such that the sheet makes contact with the buffer in both compartments. The slab gel is inverted and kept over the Whatman paper and carefully adjusted (see Note 6).
4. Preactivated membrane is placed over the gel and should be taken that no air bubbles are introduced between the gel and the membrane. Presoaked Whatman papers equilibrated in transfer buffer are placed over the membrane. Single-fold paper towels are placed over the Whatman paper to initiate capillary transfer (see Note 7).
5. The apparatus lid is placed over the paper towels and weight is applied to ensure uniform capillary transfer. After overnight transfer, the paper towels and the first Whatman filter paper are removed, and PVDF membrane is blocked with 5% milk in PBS (for mucin analysis) or 2% BSA in TBS (for mucin glycan analysis) for 1–2 h at room temperature (see Note 8).

⁵To the 225 ml of ultrapure water, 6 g of high-quality grade agarose is weighed and mixed well by swirling, in order to avoid insoluble agarose clumps. Microwave-assisted 50% power for 5–6 min enables to dissolve the agarose without over flow. 75 ml of 4× Tris buffer is added and allowed to boil briefly at the same microwave power setting.

⁶Agarose gel should be inverted prior to transfer because the bottom of the wells (and hence protein sample) is closer to the bottom side of slab gel. Further, the bottom surface will enable better transfer due to the uniform smooth surface generated during gel casting. We keep folded aluminum foil on sides to prevent direct capillary action between gel and paper towels (that can bypass the membrane and hence lower transfer efficiency).

⁷After overnight transfer, the gel along with the membrane is inverted, and the position of the wells is marked with the pencil. This is essential to prevent false labeling of the samples as well as the start position of the gel.

3.5. Probing with Antibody

1. After blocking, 1–10 µg/ml mouse monoclonal anti-MUC4 antibody 8 G7 (or other anti-mucin antibody) or rabbit polyclonal anti-mucin antiserum at 1:2,000–1:5,000 dilution in PBS is added to the PVDF membrane and incubated overnight at 4°C with gentle shaking.
2. The membrane is washed 3× for 10 min each with the 75 ml of PBST (0.05% Tween 20) followed by incubation with the HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 60 min at room temperature.
3. After the secondary antibody incubation, the membrane is washed with PBST for 3 × 10 min, followed by quick wash with the ultrapure water and prepared for the developing (see Note 10).

3.6. Lectin Blotting

1. Following overnight capillary transfer, membrane is blocked with 1% BSA in TBS at room temperature for 2 h.
2. Biotinylated lectin in TBST at 1:1,000 dilution is added to the membrane and incubated at room temperature for 3 h, with mild shaking.
3. Membrane is washed with TBST 3 × 10 min followed by incubation with the HRP-conjugated streptavidin, at 1:20,000 dilution for 60 min at room temperature.
4. Followed by washing with TBST for 3 × 5 min, the membrane is prepared for developing.

3.7. Chemiluminescence-Based Development of the Blot

1. Both the antibody and lectin probed blots are developed similarly using Pierce chemiluminescence reagent.
2. Equal volume of peroxidase solution and luminol enhancer solution is added and mixed gently. 10 ml of luminol reagent is added to cover the membrane and incubated for 1 min with gentle swirling.
3. Excess chemiluminol reagent is drained; the membrane is covered and sealed in transparent plastic and placed in the X-ray developer cassette. The blots are exposed to X-ray film for the detection of luminescence signal (see Note 9).
4. The X-ray film is developed, marked, and documented (see Note 10).

Acknowledgments

The authors on this work are supported, in part, by grants from the Department of Defense (BC074639, BC083295, and BC09742) and the National Institutes of Health (RO1 CA78590, EDNRN UO1 CA111294, RO1 CA133774, RO1 CA131944, SPORE 50 CA127297 and U54 CA163120).

⁸Both the milk and BSA are filtered using Whatman filter paper to remove the insoluble flakes, which could interfere with the detection.

¹⁰X-ray film developing should be carried out according to the signal intensity. Some antigen–antibody complex give good signal in less than minutes, whereas some only give signal only after longer exposure. With the specific negative and positive controls, false-positive and false-negative results can be ruled out.

⁹After incubation with the luminol reagent, excess reagent is removed by holding the membrane with the forceps at the diagonal corner to drain excess luminol reagent.

References

1. Kufe DW. Mucins in cancer: function, prognosis and therapy. *Nat Rev Cancer*. 2009; 9:874–885. [PubMed: 19935676]
2. Moniaux N, Andrianifahanana M, Brand RE, Batra SK. Multiple roles of mucins in pancreatic cancer, a lethal and challenging malignancy. *Br J Cancer*. 2004; 91:1633–1638. [PubMed: 15494719]
3. Rachagani S, Torres MP, Moniaux N, Batra SK. Current status of mucins in the diagnosis and therapy of cancer. *Biofactors*. 2009; 35:509–527. [PubMed: 19904814]
4. Ponnusamy MP, Lakshmanan I, Jain M, Das S, Chakraborty S, Dey P, Batra SK. MUC4 mucin-induced epithelial to mesenchymal transition: a novel mechanism for metastasis of human ovarian cancer cells. *Oncogene*. 2010; 29:5741–5754. [PubMed: 20697346]
5. Senapati S, Chaturvedi P, Chaney WG, Chakraborty S, Gnanapragassam VS, Sasson AR, Batra SK. Novel INTERaction of MUC4 and galectin: potential pathobiological implications for metastasis in lethal pancreatic cancer. *Clin Cancer Res*. 2011; 17:267–274. [PubMed: 21059814]
6. Belisle JA, Horibata S, Jennifer GA, Petrie S, Kapur A, Andre S, Gabius HJ, Rancourt C, Connor J, Paulson JC, Patankar MS. Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes. *Mol Cancer*. 2010; 9:118. [PubMed: 20497550]
7. Senapati S, Das S, Batra SK. Mucin-interacting proteins: from function to therapeutics. *Trends Biochem Sci*. 2010; 35:236–245. [PubMed: 19913432]
8. Kinlough CL, Poland PA, Gendler SJ, Mattila PE, Mo D, Weisz OA, Hughey RP. Core-glycosylated mucin-like repeats from MUC1 are an apical targeting signal. *J Biol Chem*. 2011; 286(45):39072–39081. [PubMed: 21937430]
9. Schägger H. Tricine-SDS-PAGE. *Nat Protoc*. 2006; 1:16–22. [PubMed: 17406207]
10. Warren CM, Krzesinski PR, Greaser ML. Vertical agarose gel electrophoresis and electroblotting of high-molecular-weight proteins. *Electrophoresis*. 2003; 24:1695–1702. [PubMed: 12783444]
11. Burdick MD, Harris A, Reid CJ, Iwamura T, Hollingsworth MA. Oligosaccharides expressed on MUC1 produced by pancreatic and colon tumor cell lines. *J Biol Chem*. 1997; 272:24198–24202. [PubMed: 9305871]
12. Argüeso P, Guzman-Aranguez A, Mantelli F, Cao Z, Ricciuto J, Panjwani N. Association of cell surface mucins with galectin-3 contributes to the ocular surface epithelial barrier. *J Biol Chem*. 2009; 284(34):23037–23045. [PubMed: 19556244]

Fig. 1.

Schematic flow chart for the mucin glycan analysis. Cells lines were trypsinized, and mechanical disruption by syringe passage will be carried out. After freezing and thawing, immunoprecipitation and Western analysis of mucin glycans were carried out. For tissues liquid nitrogen was frozen and grounded with pestle and mortar, followed by the addition of RIPA and continued with the steps as discussed for the cell line mucin glycan analysis. Glycoprotein-specific antibody and lectins were tabulated, also an example for the mucin-associated glycotopes SLe^c, SLe^a, SLe^x, and SLe^A (from *left to right*) and ConA lectin staining of alpha-linked mannose attached to the *N*-linked glycan of mucin.