Lectin-Based Detection of Carbohydrates with Near-Infrared Fluorophores

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Abstract

Alterations in glycosyl epitopes play key roles in tumor progression. Epidermal growth factor receptor (EGFR) is a well-known glycoprotein that is currently targeted for anticancer therapy.[1] We used Con A, WGA and UEA-I to detect the presence of carbohydrates on EGFR in Western blot format and employed near-infrared (IR) fluorophores as the detection mechanism. IR fluorescent detection bypasses the need for colorimetric or chemiluminescent reagents and produces more quantitative results than enzymatic systems. First, the IR detection method using Western blot format was validated on a set of glycosylated proteins and non-glycosylated proteins (negative controls), and detected with IRDye™ 800CW-conjugated streptavidin using a laser imager appropriate for IR wavelengths. High sensitivity (4-8 ng) was achieved with this system. Negative controls and PNGase F controls demonstrated that the detection methodology was highly specific. Next, we also found that glycoproteins could be detected and identified by a multiplexed fluorescent methodology. Con A was directly conjugated with IRDye™ 800CW and used to detect glycosylated proteins in lysates of A431 cells in Western blot format. The blot was simultaneously probed with a primary antibody against EGFR, a protein that contains multiple N-linked glycosylation sites. AlexaFluor® 680 secondary antibody was used to visualize binding of the EGFR primary antibody. This two-color technique allowed for simultaneous determination of protein identity and glycosylation state on a single Western blot. Lastly, we show that EGFR binds to Con A and WGA, but not to UEA-I in human epidermal cancer cell lysates. Thus, the IRDye™ 800CW-conjugated lectin detection system has the advantages of producing high sensitivity as well as specificity from using a two-colored near-infrared fluorophore system and can be used to detect alterations in glycosyl epitopes that are commonly associated with tumor progression.

Introduction

Visible fluorescence is not commonly used for detection of proteins in Western format, since the high autofluorescence background of blotting membranes dramatically affects detection sensitivity and makes the technique impractical. However, blotting membranes have much lower autofluorescence in the near-infrared region of the spectrum. The resulting improvement in signal to noise makes it possible to achieve high sensitivity with the convenience of direct fluorescent detection. The Odyssey® Infrared Imaging System is optimized for imaging of near-infrared fluorescence at 700 and 800 nm. We used this instrument to characterize glycoproteins in Western blot format, using labeled lectins and near-infrared fluorescent detection.

Materials & Methods

Odyssey® Blocking Buffer (LI-COR® Biosciences; Lincoln, NE) was used in all experiments. Fluorescent blots and Coomassie stained gels were imaged with the Odyssey Infrared Imaging System (LI-COR Biosciences) at 700 and 800 nm.

Detection of purified glycoproteins

For sensitivity determinations, two-fold dilutions were electrophoresed and transferred to nitrocellulose. Blots were incubated with biotinylated Con A diluted 1:1000, followed by IRDye™ 800CW streptavidin diluted 1:10,000. Alternatively, Con A was directly conjugated with amine-reactive IRDye™ 800CW (LI-COR Biosciences) with a dye/protein ratio of 1.7 and used at 1:1000.

Purified proteins (20 ug) were digested with Peptide: N-Glycosidase F, which cleaves between the innermost GlcNAc and asparagine residues of N-linked glycoproteins. One gel was stained with Coomassie and another transferred to nitrocellulose. Blots were detected with biotinylated Con A and IRDye™ 800CW streptavidin.

Characterization of EGFR in cell lysates

A431 cell lysates were digested with PNGase F. One gel was stained with Coomassie, and another transferred to nitrocellulose. Blots were detected with anti-human EGFR (Biosource, Cat. # AHR5062) then with AlexaFluor® 680 goat-anti-mouse (Molecular Probes, Cat. # A21057) and IRDye™ 800CW-Con A.

Con A, WGA and UEA-I were conjugated with amine-reactive IRDye™ 800CW. Normal epidermal, cancer epidermal (A431), normal liver, and cancer liver (HepG2) lysates were detected with anti-EGFR antibody as above. Blots were also incubated with IRDye™ 800CW-labeled Con A, WGA, or UEA I.

Results

Sensitivity of biotinylated Con A/IRDye™ 800CW-streptavidin and IRDye™ 800CW-Con A

Biotinylated Con A and infrared-labeled streptavidin were used to visualize binding of Con A to sugar moieties on known glycoproteins. As an alternative, Con A was conjugated with IRDye™ 800CW by standard amine-directed labeling, generating a directly labeled lectin. Sensitivity of these two detection methods was determined in Western blot format (Figure 1).

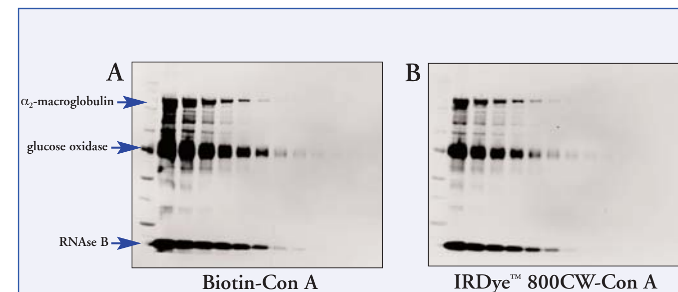

Figure 1. Sensitivity of biotinylated Con A/IRDye™ 800CW streptavidin and IRDye™ 800CW-Con A. Western blot of two-fold serial dilutions of α_2-macroglobulin (180 kD), glucose oxidase (82 kD), and RNAse B (17 kD) from 2 µg to 244 pg. The limit of detection for glucose oxidase was 4 ng (lane 10) with biotinylated Con A/IRDye™ 800CW streptavidin (panel A), and 8 ng (lane 9) with IRDye™ 800CW-Con A (panel B).

Detection of EGFR in A431 cell lysates using human anti-EGFR/AlexaFluor® 680 antibody and IRDye™ 800CW-Con A

Next, we devised a multiplexed method to glean information about both glycosylation state and target protein identity in complex protein samples. Epidermal growth factor receptor (EGFR) was analyzed in cell lysates using anti-EGFR antibody and Alexa Fluor® 680 secondary antibody in combination with IRDye™ 800CW-Con A (Figure 3). PNGase F was again used to confirm specificity. This experiment showed that anti-EGFR antibody and Con A co-localize on the blot – evidence that EGFR is glycosylated. The shift in mobility of EGFR following PNGase F treatment indicates that N-linked sugars were removed by the glycosidase, providing further confirmation.

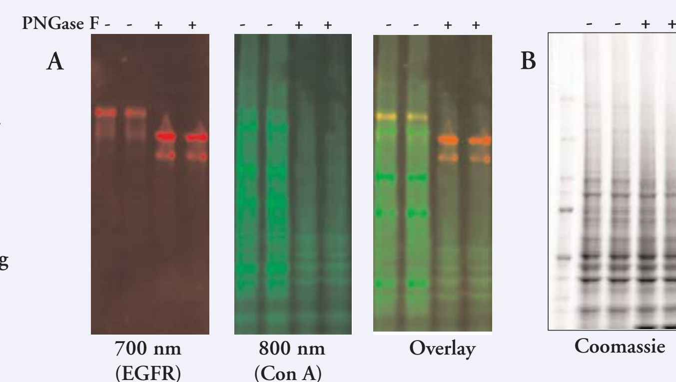

Figure 3. Detection of EGFR in A431 cell lysates using human anti-EGFR/AlexaFluor® 680 antibody and IRDye™ 800CW-Con A. A) Each panel shows duplicate lanes of A431 cell lysate, untreated or digested with PNGase F. EGFR protein was detected in the 700 nm channel (red), glycoproteins were detected in the 800 nm channel with dye-conjugated Con A (green), and the overlay panel shows a composite image. Yellow band (overlap of red and green) indicates glycosylated EGFR. B) Coomassie stain of a replicate gel, confirming equal loading of samples.

Detection of purified glycoproteins using biotinylated Con A/IRDye™ 800CW-streptavidin

We then used PNGase F, a glycosidase which removes terminal and N-linked sugars, to evaluate the specificity of Con A binding. A shift in migration in a digested sample indicates that N-linked oligosaccharides were removed. Appropriate band shifts were observed for α2-macroglobulin, glucose oxidase and transferrin in Coomassie stained samples; no shift was observed for phosphorylase and BSA, which are not glycoproteins (Figure 2A). Data was consistent with published reports.

When detected with biotin-Con A and IRDye™ 800CW-streptavidin, α2-macroglobulin and glucose oxidase showed strong bands in undigested samples and the absence of corresponding bands in digested samples, indicating that these proteins contain carbohydrate residues capable of binding Con A (Figure 2B). Phosphorylase and BSA were not detected. Transferrin showed some binding to Con A after digestion with PNGase F; it is possible that it contains an oligosaccharide coupled via O-linkage, which would not be digested by PNGase F. Transferrin is predicted to have an O-linkage in addition to N-linkage[2]. *Note: Lectin blots do not provide proof of a carbohydrate structure and are not a substitute for more specific and quantitative analytical approaches.*[3]

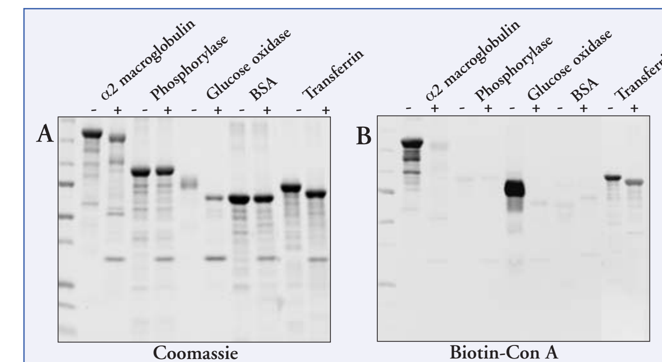

Figure 2. Detection of purified glycoproteins using biotinylated Con A/IRDye™ 800CW streptavidin. A) Coomassie stain of undigested (-) and PNGase F-digested (+) proteins. B) Western blot showing undigested and PNGase F-digested proteins. Blot was probed with biotinylated Con A and detected with IRDye™ 800CW streptavidin.

Characterization of EGFR in normal and cancer cell lysates using labeled lectins

Alterations in glycosyl epitopes can play key roles in tumor progression, and EGFR is a popular target for anticancer therapy. Probing with additional IRDye™-conjugated lectins could be a simple way to further dissect the sugar composition of proteins such as EGFR. To test this, we used dye-conjugated WGA and UEA-I for detection (Figure 4).

Con A and WGA bound to EGFR (yellow bands, panels B and C), but UEA-I did not (panel D). Con A binds to α-D-mannose, α-D-glucose and to a lower affinity, α-N-GlcNacs[4,5]; WGA binds ß-GlcNAcs and sialic acid[6,7]; and UEA-I binds α-fucose[8,9]. EGFR was highly expressed in cancer epidermal tissue, but was not detected in normal epidermal or liver tissue. Very different lectin banding patterns were observed between normal and cancer tissue lysates, suggesting that infrared-labeled lectins might be a useful tool for differentiation of normal and cancer cells.

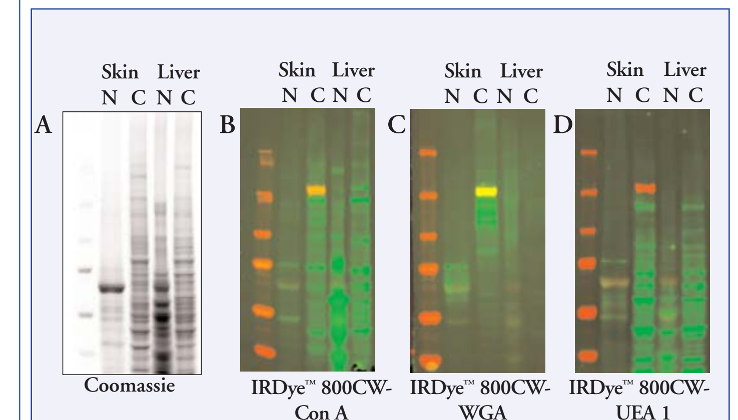

Figure 4. Characterization of EGFR in normal and cancer cell lysates using labeled lectins. Lysates of normal (N) and cancer (C) tissues from human epidermis and liver. Gel was stained with Coomassie. B-D) Western blots showing two-color detection of EGFR using mouse anti-human EGFR + AlexaFluor® 680 goat-anti-mouse (red), and IRDye™ 800CW (green) conjugated to Con A, WGA, and UEA-I.

Summary

- Detection of glycoproteins with infrared dyes and the Odyssey® System was accurate and highly sensitive, with detection limits in the 4-8 ng range.
- A multiplexed fluorescent method was developed. This method yields information about glycosylation state (using lectin-based detection) and also confirms the identity of the glycoprotein (using a specific antibody). Carbohydrate composition can be dissected using multiple labeled lectins.
- Biotinylated lectins and IRDye™ 800CW streptavidin are a ready-to-use solution for carbohydrate detection, and directly-labeled lectins can also be generated.

References

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