Biochemical and Biophysical Research Communications 500 (2018) 765–771

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Specificity and utility of SubB2M, a new *N*-glycolylneuraminic acid lectin

Jing Wang [a, 1], Lucy K. Shewell [a, 1], Adrienne W. Paton [b], James C. Paton [b], Christopher J. Day [a, **], Michael P. Jennings [a, *]

[a] Institute for Glycomics, Griffith University, Gold Coast, QLD 4222, Australia
[b] Research Centre for Infectious Diseases, Department of Molecular and Biomedical Science, University of Adelaide, S.A., 5005, Australia

ARTICLE INFO

Article history:
Received 14 April 2018
Accepted 17 April 2018
Available online 26 April 2018

Keywords:
N-glycolylneuraminic acid
Neu5Gc
Lectin
SubB
Cancer

ABSTRACT

The B subunit of the subtilase cytotoxin (SubB) recognises *N*-glycolylneuraminic acid (Neu5Gc) containing glycans, the most prominent form of aberrant glycosylation in human cancers. We have previously engineered SubB by construction of a $SubB_{\Delta S106/\Delta T107}$ mutant (SubB2M) for greater specificity and enhanced recognition of Neu5Gc containing glycans. In this study, we further explore the utility of SubB2M as a Neu5Gc lectin by showing its improved specificity and recognition for Neu5Gc containing glycans over the wild-type SubB protein and an anti-Neu5Gc IgY antibody in a *N*-acetylneuraminic acid (Neu5Ac)/Neu5Gc glycan array and by surface plasmon resonance. Far-western blot analysis showed that SubB2M preferentially binds to bovine serum glycoproteins over human serum glycoproteins. SubB2M was also able to detect Neu5Gc containing bovine glycoproteins spiked into normal human serum with greater sensitivity than the wild-type SubB and the anti-Neu5Gc IgY antibody. These results suggest that SubB2M will be a useful tool for the testing of serum and other bodily fluids for cancer diagnosis and prognosis.

1. Introduction

The Shiga toxigenic *Escherichia coli* (STEC) Subtilase cytotoxin (SubAB) targets α2-3-linked *N*-glycolylneuraminic acid (Neu5Gc) via its pentameric B-subunit SubB [1,2]. Neu5Gc terminating glycans are not expressed at significant levels on healthy human tissues [3–6] as humans produce an inactive cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH) enzyme [7]. However, Neu5Gc containing glycans are the most prominent form of aberrant glycosylation in human cancers and can be explained by dietary intake of red meat and dairy products leading to the absorption of Neu5Gc [8].

In recent work we engineered the SubB protein to increase specificity and selectivity for Neu5Gc containing glycans by limiting Neu5Ac recognition and by broadening the types of Neu5Gc linkages recognised [9]. Of the six mutant SubB proteins produced, the $SubB_{\Delta S106/\Delta T107}$ mutant (SubB2M) fulfilled the aim of less Neu5Ac recognition and broadened Neu5Gc linkage recognition [9], including binding to Neu5Gcα2-6 containing glycans, possible cancer antigens [10]. The improved SubB2M lectin offers a potential new tool for the testing of serum and other bodily fluids from individuals with or suspected of having cancer. To further confirm the suitability of SubB2M as a tool for Neu5Gc detection in both laboratory and diagnostic settings we tested the protein with a new Neu5Ac/Neu5Gc paired structure glycan array and used the lectin in immunoassays, including, far-western blots and in Surface Plasmon Resonance (SPR) to detect Neu5Gc containing glycoproteins.

2. Materials and methods

2.1. Expression and purification of wild-type SubB and SubB2M

The wild-type SubB and SubB2M recombinant proteins were expressed and purified as previously described [9].

* Corresponding author.
** Corresponding author.
E-mail addresses: c.day@griffith.edu.au (C.J. Day), m.jennings@griffith.edu.au (M.P. Jennings).
[1] These authors contributed equally.

https://doi.org/10.1016/j.bbrc.2018.04.151

2.2. Glycan array analysis of wild-type SubB and SubB2M

Neu5Ac/Neu5Gc glycan array slides were purchased from Z-Biotech (Aurora, Colorado, USA). The arrays were performed as per the manufacturer's instructions with the following modifications. For each subarray 2 μg of SubB proteins were pre-complexed with anti-His tag antibody (Cell signalling) and Alexa555 secondary and tertiary antibodies (rabbit anti-mouse; goat anti-rabbit; Thermo Scientific) at a ratio of 2:1:0.5:0.25 in a final volume of 100 μL. This 100 μl antibody protein complex was added to the silicon gene frame (Z-Biotech) without a coverslip. Washing was performed as previously described [11] and scanned using an Innoscan 1100AL scanner and analysed using Mapix analysis software as described in Day et al. 2017 [12].

2.3. Surface plasmon resonance of SubB with Neu5Ac/Neu5Gc glycan pairs

Surface plasmon resonance (SPR) was run using the Biacore T200 system (GE) as described previously [9]. Briefly, SubB, SubB2M and anti-Neu5Gc IgY (Biolegend) were immobilized onto flow cell 2–4 of a series S sensor chip CM5 (GE) using the NHS capture kit and flow cell 1 was run as a blank immobilization. Neu5Ac/Neu5Gc pairs were purchased from Chemily Glycoscience (Atlanta, GA). Glycans were used across a five-fold dilution series at a maximum concentration of 20 μM. Analysis was run using single cycle analysis and double reference subtraction on the Biacore T200 evaluation software.

2.4. Far-western blot analysis

2.4.1. Glycoproteins

Each glycoprotein was diluted to 5 mg/ml and treated with 20 U of α2-3,6,8,9 neuraminidase (sialidase) A (New England BioLabs) in a final volume of 50 μl of 1 × GlycoBuffer 1 (New England BioLabs). 10 μg of human and bovine α1-acid glycoproteins (AGP), fetuin and asialofetuin (Sigma-Aldrich) and native bovine MUC-1 (Creative Bio-labs) (either with or without neuraminidase treatment) were combined with 1X NuPAGE sample buffer and 5% (v/v) β-mercaptoethanol, heated at 99 °C for 10mins and loaded into an SDS-polyacrylamide gel (NuPage 4–12% Bis-Tris gel, Invitrogen). The separated proteins were then transferred to nitrocellulose membrane for far-western blot analysis. The membranes were blocked with 1% fish gelatin (Sigma-Aldrich) in phosphate buffered saline (PBS) for 1 h and then washed once with PBS/0.05% Tween-20 (PBST). Then 1 μg/ml of wild-type SubB or SubB2M or chicken anti-Neu5Gc IgY antibody (1:10 000) (BioLegend) was used to detect Neu5Gc on glycoproteins. All membranes were washed in 1 × PBST three times. Blots were probed with monoclonal anti-polyhistidine-Alkaline Phosphatase mouse antibody (1:10 000) (Sigma-Aldrich) to detect binding of SubB and SubB2M or with rabbit anti-chicken IgY antibody (1:10 000) (Sigma-Aldrich) to detect binding of the anti-Neu5Gc antibody, both diluted in PBS. The membranes were washed with PBST three times, then developed with NBT/BCIP solution (Sigma-Aldrich) for 10 min.

2.4.2. Sera from human and bovine

Serum samples were purchased from commercial suppliers: human (Sigma-Aldrich) and bovine (Sigma-Aldrich). Serum samples were diluted to 5 mg/ml and treated with 20 U of α2-3,6,8,9 neuraminidase (sialidase) A in a final volume of 50 μl of 1 × GlycoBuffer 1. Samples were then incubated at 37 °C for 2 h, as previously described [13]. 10 μg of total serum protein (either with or without neuraminidase treatment) was combined with 1X NuPAGE sample buffer and 5% (v/v) β-mercaptoethanol, heated at 99 °C for 10mins and loaded into an SDS-polyacrylamide gel. The separated serum proteins were then transferred to nitrocellulose membrane for far-western analysis. Membranes were probed with SubB, SubB2M and anti-Neu5Gc antibody and detected as described above for glycoproteins. Membranes were also probed with 1 μg/ml of *Sambucus nigra* agglutinin (SNA)-1-alkaline phosphatase conjugate (EY Laboratories) in PBS to detect sialic acids on glycoconjugates as an additional positive control.

2.5. Surface plasmon resonance of SubB proteins with Neu5Gc spiked human serum

Surface plasmon resonance (SPR) was run using the Biacore S200 system (GE) as described previously [14]. Briefly, SubB, SubB2M and anti-Neu5Gc IgY were immobilized onto flow cell 2–4 of a series S sensor chip CM5 (GE) using the NHS capture kit and flow cell 1 was run as a blank immobilization. Bovine α1-Acid glycoprotein and MUC1 were spiked into 1% normal human serum (NHS; Sigma-Aldrich) starting at 1μM. The protein was diluted 1:2 in 1% NHS across 5–6 dilutions and 1% NHS was used as the zero concentration control. Analysis was run using multi-cycle analysis and double reference subtraction on the Biacore S200 evaluation software.

3. Results

3.1. Glycan array and SPR analysis of wild-type SubB and SubB2M

To further assess the specificity of SubB2M for Neu5Gc and underlying glycan linkages, a Neu5Ac/Gc array from Z-Biotech was employed. The Z-Biotech array features 40 Neu5Ac/Gc pairs and one with both Neu5Ac and Neu5Gc on the one glycan, counted as Neu5Gc for analysis, with both linear and branched structures (Fig. S1). Binding to Neu5Gc structures was preferred by the wild-type SubB, but there were four out of 40 Neu5Ac glycans that were bound with greater than 5000 relative fluorescent units (RFU) above background and 14 out of 41 Neu5Gc structures that had binding below 5000 RFU (Fig. 1A and B). The SubB2M protein, however, showed no binding to Neu5Ac glycans with greater than 5000 RFU above background and only five out of 41 Neu5Gc structures had binding below 5000 RFU. Only eight of 40 Neu5Ac glycans showed any binding above background for SubB2M (Fig. 1A and C). It was also noted that SubB2M was better able to recognise fucosylated Neu5Gc containing glycans, sialyl-Lewis A and X, with improved binding to structures 3, 6, 9, 14, 17, 20, 23, 29 and 35 (Fig. 1B and C, Fig. S1). These are all structures containing Neu5Gc only present on the same terminal end as the sub-terminal fucosylation.

SPR analysis was performed on three sets of Neu5Ac/Gc pairs; α2-3Neu5Ac/Gc Lacto-N-neotetraose (Ac/Gc-SLNnT), Ac/Gc sialyl-Lewis X (Ac/Gc-SLeX) and Ac/Gc monosialylated ganglioside 1 (Ac/Gc-G_{M1}) (Table 1; Fig. S2). SubB2M and the anti-Neu5Gc IgY did not bind to any of the Neu5Ac structures, while wild-type SubB recognised the Ac-SLNnT with a K_D of 2.68 μM (Table 1). Wild-type SubB bound Gc-SLeX with a similar K_D (2.06 μM; Table 1) to the recognition of Ac-SLNnT and the addition of the branched fucose to the Gc-SLNnT (Gc-SLeX) reduced the K_D by 300-fold. SubB2M bound Gc-SLeX with a K_D (94.85nM), 21.7-fold better than the wild-type SubB but still 31-fold reduced affinity compared to the non-fucosylated Gc-SLNnT (Table 1). The anti-Neu5Gc IgY did not bind to Gc-SLeX at a maximum concentration of 20 μM (Table 1). The Gc-G_{M1} was bound by all three proteins with 20–100 fold less affinity than the Gc-SLNnT demonstrating that all three of these proteins prefers Neu5Gc presented as the terminal sugar (exo) rather than on a internal branch (endo).