Development of a High-Specificity Affinity Reagent for *N*-Glycosylation Detection and Enrichment

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Abstract

Glycans play crucial roles in nearly every aspect of biological processes, and their distinct properties make them appealing as disease biomarker targets. However, due to their highly branched and variably linked nature, glycans pose a challenge for their detection, purification, and structural analysis. Although advanced analytical techniques and instrumentation have been developed, there is still a great need for reagents with well-defined carbohydrate specificity and high affinity that can be used to interrogate and enrich biological samples. Lectenz Bio has been engineering glycan-processing enzymes and glycan-binding proteins into high-affinity glycan-binding reagents with tunable specificities. Here, we report the development of an asparagine-linked glycan (*N*-glycan) reagent engineered from mouse Fbs1 (Fbx2), a component of the E3 ubiquitin ligase complex. We demonstrate that the reagent binds specifically to *N*-glycosylated peptides and proteins and not to the corresponding non-glycosylated peptides and proteins.

Characterization of Fbs1 and mutants

The sugar-binding domain of Fbs1, the candidate *N*-glycan affinity reagent, can be readily expressed on the surface of yeast and recombinantly in *E. coli.* Fbs1 binds specifically to *N*-glycosylated peptides and proteins as shown by yeast display (**Fig. 1**), bio-layer interferometry (**Fig. 2**), glycan microarray screening (**Fig. 3**), Western blot (**Fig. 5**), and affinity chromatography (**Fig. 6**). One of the engineered mutants, Fbs1-7, exhibits enhanced binding to complex *N*-glycopeptides/proteins.

Yeast Display Assay

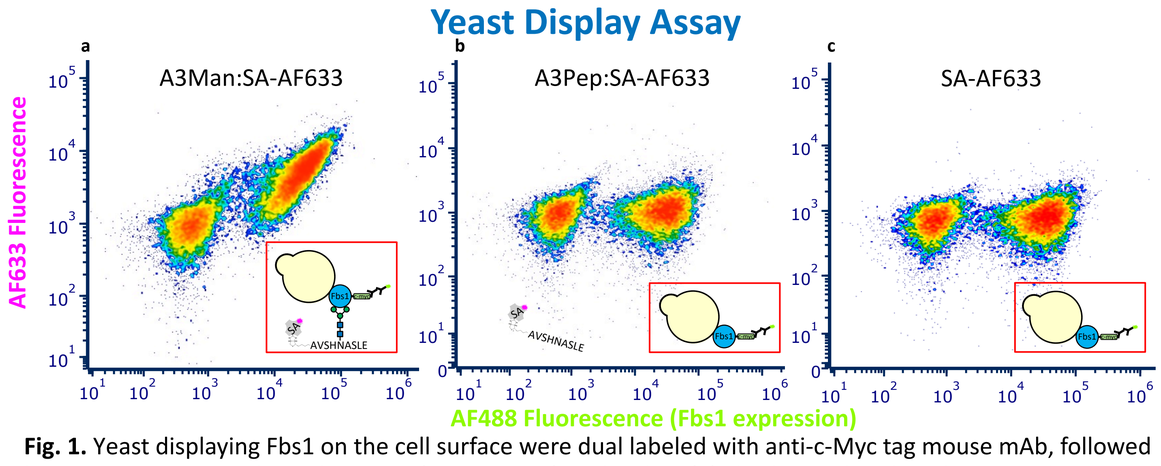


Fig. 1. Yeast displaying Fbs1 on the cell surface were dual labeled with anti-c-Myc tag mouse mAb, followed by goat anti-mouse-Alexa Fluor 488 (GaM-AF488) and either (**a**) the biotinylated *N*-glycopeptide A3Man precomplexed with streptavidin-Alexa Fluor 633 (SA-AF633), (**b**) the biotinylated peptide A3Pep precomplexed with SA-AF633, or (**c**) the SA-AF633-only control. The increase in AF633 fluorescence of yeast binding to glycopeptide, but not non-glycosylated peptide, demonstrates that Fbs1 will be amenable to direct evolution using yeast display methods.

Bio-Layer Interferometry

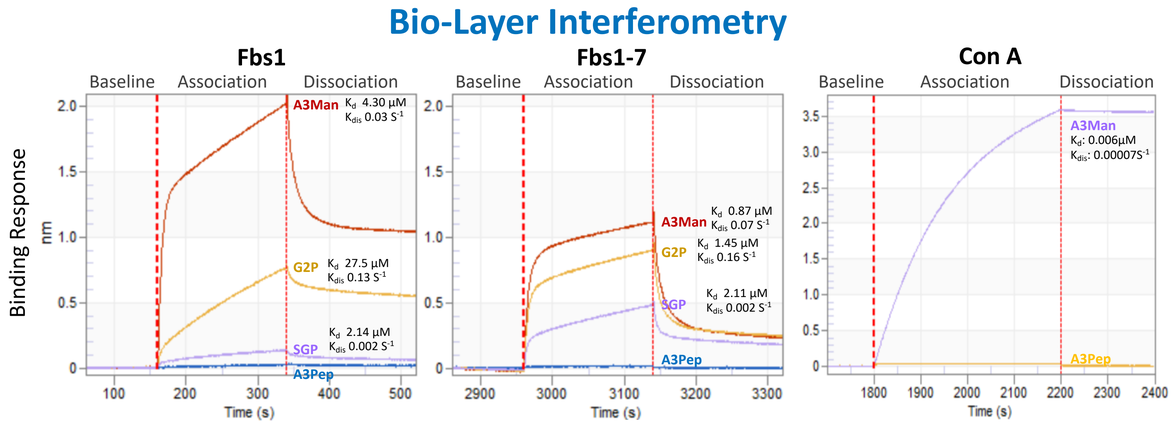
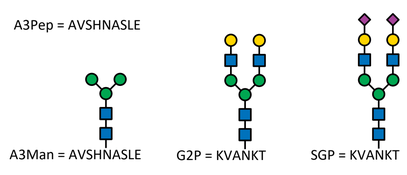


Fig. 2. Binding of Fbs1, Fbs1-7, and Con A to biotinylated *N*-glycopeptides (A3Man, G2P, SGP) and a biotinylated peptide (A3Pep) immobilized onto streptavidin BLI biosensors. Preliminary transient binding constants indicate Fbs1-7 has enhanced binding response and/or affinity to complex *N*-glycopeptides. Con A is a lectin which binds preferentially to high mannose *N*-glycans.



Glycan Microarray Screening

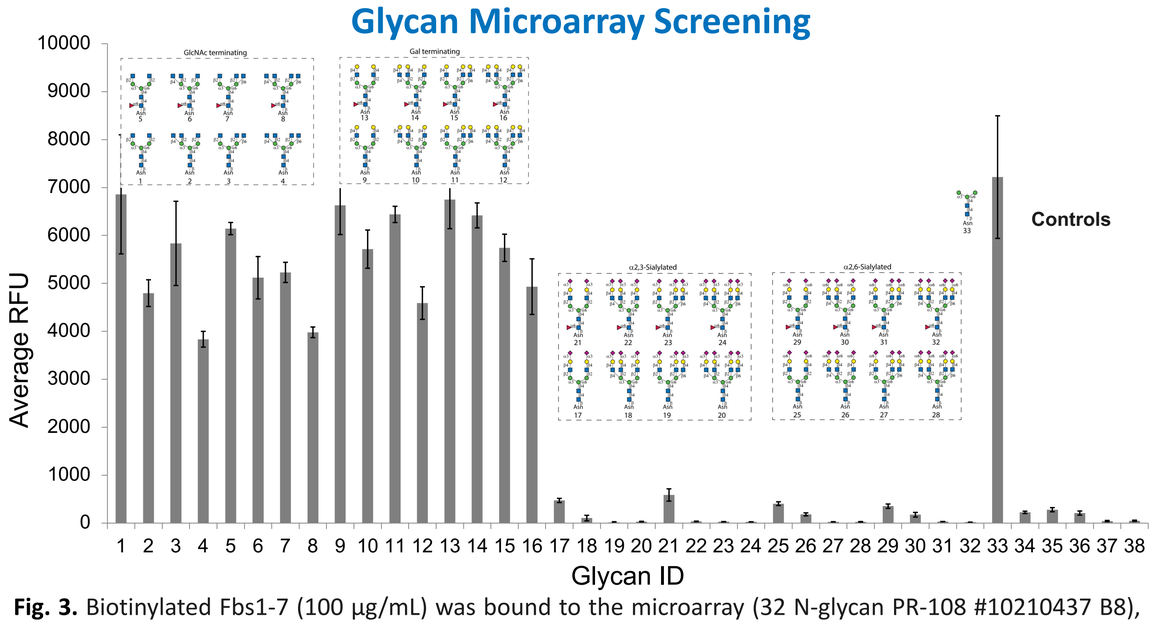


Fig. 3. Biotinylated Fbs1-7 (100 µg/mL) was bound to the microarray (32 N-glycan PR-108 #10210437 B8), followed by 0.5 µg/mL of a streptavidin-Cy5 conjugate. Fbs1-7 shows a preference for terminal GlcNAc, Gal, and Man *N*-glycans over terminally sialylated glycans, and does not bind glycans that are not *N*-linked (controls). Glycan binding assays were performed by the National Center for Functional Glycomics (NCFG).

Computational Modeling

Using our proprietary platform for computationally guided directed evolution, we aim to engineer an array of *N*-glycan-specific binding proteins for the detection and enrichment of all forms of *N*-glycosylation. Molecular Dynamics (MD) simulations of Fbs1-*N*-glycan complex were performed using coordinates for the sugar-binding domain of murine Fbs1 taken from the wild-type crystal structure (PDBID: 1UMI) with the crystallographic chitobiose ligand being replaced by the core *N*-glycan structure $Man_3GlcNAc_2$ (**Fig. 4**). The contribution of per-residue binding energy of each amino acid were then calculated.

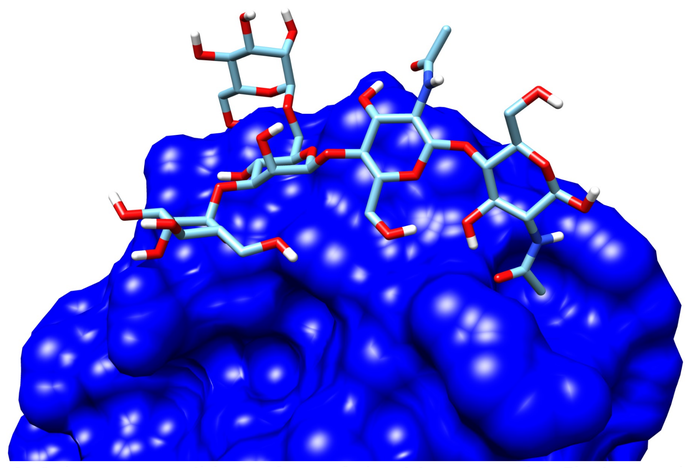


Fig. 4. Solvent accessible surface of the *Mus musculus* Fbs1 complexed with the *N*-glycan structure $Man_3GlcNAc_2$ (generated by GLYCAM).

Applications

Using computational analysis and/or site-directed mutagenesis, a number of Fbs1 variants will be generated and tested in a variety of detection and isolation assays.

Western Blot

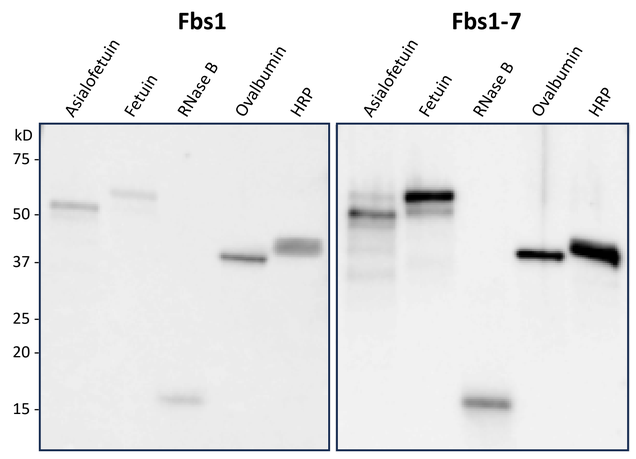


Fig. 5. Glycoproteins (Asialofetuin, Fetuin, RNase B, Ovalbumin, and Horse Radish Peroxidase) were separated by SDS-PAGE, blotted to nitrocellulose membrane, and probed with His-tagged Fbs1 or Fbs1-7, followed by an anti-His-tag antibody-HRP conjugate. The results demonstrate that both probes bind to all the glycoproteins, with Fbs1-7 having >5 fold improved sensitivity over native Fbs1.

Affinity Chromatography

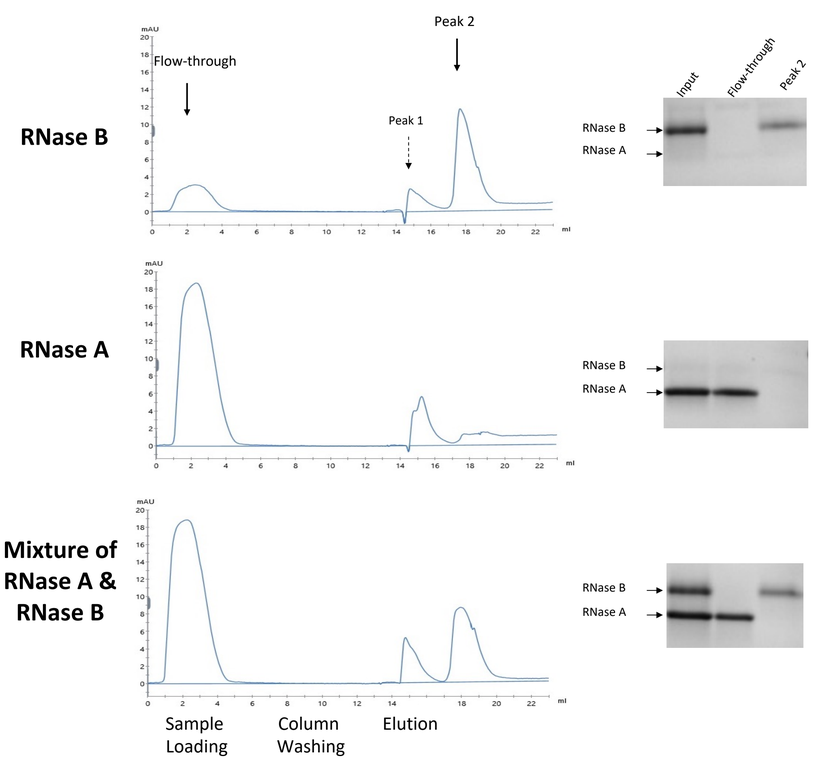


Fig. 6. Fbs1 (5 mg) was chemically coupled to a 1 mL Sepharose column. A glycoprotein, RNase B, and its non-glycosylated counterpart, RNase A, as well as a 1:1 mixture of RNase A and RNase B were respectively loaded to the column in buffer. Bound protein was eluted non-competitively. Collected Flow-through (unbound fraction) and elution Peak 2 were analyzed by SDS-PAGE to confirm separation of RNase B from RNase A. Note that "Peak 1" is an artifact resulting from the change from loading buffer to the elution buffer. As it was previously determined not to contain any protein, "Peak 1" is not shown on the SDS-PAGE gel.

Future Directions

In addition to Fbs1, two other murine F-box only proteins, FBXO6 and FBXO27, whose predicted structures are similar to Fbs1's, may be used as scaffolds for engineering a suite of *N*-glycan affinity reagents.

Acknowledgements

Lectenz Bio team

National Institutes of Health
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