

Photoactivatable Glycopolymers for the Proteome-Wide Identification of Fucose- α (1-2)-Galactose Binding Proteins

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S Supporting Information

ABSTRACT: Although fucose- α (1-2)-galactose (Fuca(1-2)Gal)-containing glycans have been implicated in cognitive processes such as learning and memory, their precise molecular mechanisms are poorly understood. Here we employed the use of multivalent glycopolymers to enable the first proteome-wide identification of weak affinity, low abundance Fuca(1-2)Gal glycan-binding proteins (GBPs). Biotin-terminated glycopolymers containing photoactivatable cross-linking groups were designed to capture and enrich GBPs from rat brain lysates. Candidate proteins were tested for their ability to bind Fuca(1-2)Gal, and the functional significance of the interaction was investigated for the synaptic vesicle protein SV2a using a knockout mouse system. The results suggest a role for SV2a-Fuca(1-2)Gal interactions in SV2a trafficking and synaptic vesicle recycling. More broadly, our studies outline a general chemical approach for the systems-level discovery of novel GBPs.

Glycan-binding interactions play important roles in many complex physiological processes, including the immune response, tumor metastasis, and viral infection.¹ The distinct arrays of glycans presented on cell surfaces are recognized by various glycan-binding proteins (GBPs) or lectins. The ability of GBPs to bind and often cluster glycan-presenting receptors can have important consequences for processes such as cell adhesion, migration, and gene regulation.^{1,2}

Despite their importance, mammalian GBPs that interact with specific glycans of interest have been challenging to identify.³ The weak affinities of many glycan–protein interactions ($K_{\text{assoc}} = 10^3\text{--}10^6\text{ M}^{-1}$) have complicated efforts to capture and study endogenous GBPs. Most of the well-characterized lectins are derived from plants (e.g. concanavalin A) or were discovered through classical biochemical purification or structural homology to known lectins. Notably, general systems-level approaches for the proteome-wide identification of GBPs have been lacking. The development of such methods is critical for elucidating the structure–function relationships of carbohydrates and understanding the diverse roles of GBPs. Here we address these challenges through the synthesis and characterization of chemical probes for the discovery of novel mammalian GBPs.

We focused on targeting neuronal GBPs that interact with glycans containing the fucose- α (1-2)-galactose (Fuca(1-2)Gal) motif. Fuca(1-2)Gal is found on the nonreducing termini of

many glycans and has been implicated in neuronal development, learning, and memory.⁴ For example, treatment of animals with 2-deoxy-D-galactose (2-dGal), a compound that disrupts the formation of Fuca(1-2)Gal linkages, caused reversible amnesia^{4a} and interfered with the maintenance of long-term potentiation,^{4b} an electrophysiological model of learning and memory. Moreover, previous work from our laboratory has suggested the existence of both Fuca(1-2)Gal GBPs and glycoproteins as well as their involvement in neurite outgrowth and synaptogenesis.^{4c,d} Understanding the molecular mechanisms underlying the activity of Fuca(1-2)Gal sugars will require identification of the key molecular components involved. However, no Fuca(1-2)Gal GBPs have been identified from the mammalian brain.

Our early attempts to isolate Fuca(1-2)Gal GBPs employed monovalent ligands conjugated to agarose beads with or without photoactivatable cross-linking groups. Although a few candidate GBPs were successfully detected, this approach failed to capture sufficient quantities of protein for mass spectrometry (MS) analysis. To overcome these challenges, we designed synthetic glycopolymers that contain several key elements (Figure 1). First, we exploited multivalent sugar epitopes to augment weak glycan–GBP interactions.⁵ Second, we incorporated a photo-reactive nitrophenylazide moiety into the glycopolymer to enable covalent cross-linking of the associated proteins. Lastly, we end-functionalized the glycopolymers with a biotin handle to facilitate affinity enrichment and identification of the GBPs.

To synthesize the glycopolymers, we explored cyanoxyl (OCN)-mediated free radical polymerization chemistry because it affords water-soluble polymers of controlled length and narrow polydispersity.⁶ The Fuca(1-2)Gal epitope or a control ligand lacking the disaccharide was incorporated into acryloyl-functionalized monomers **1** and **2**, respectively (Figure 1). We chose a nitrophenylazide cross-linking agent for monomer **3** because this group has been successfully applied to proteins at membrane interfaces.⁷ For end-labeling the polymers with a biotin moiety, we employed the arylamine initiator **4** developed by Chaikof et al.^{6b}

Fuca(1-2)Gal monomer **1** was prepared using several one-pot, multistep reactions (Scheme S1). Briefly, disaccharide **5**⁸ was treated with (phenylthio)trimethylsilane in the presence of zinc iodide, deprotected with TBAF, and the resulting C-6 hydroxyl group acetylated to afford thioglycoside **6**. Although coupling of 3-azido-1-propanol to this thioglycoside using NIS/AgOTf in

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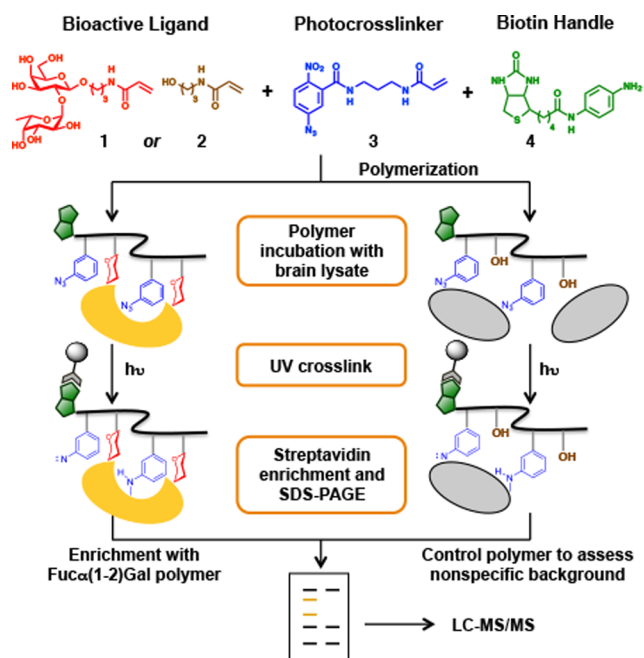
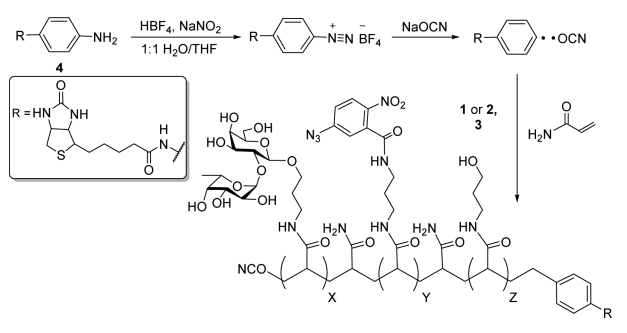


Figure 1. Strategy for the systems-level, proteome-wide identification of GBPs.

CH_2Cl_2 yielded an anomeric α/β mixture, acetonitrile as the solvent promoted formation of the β -linked glycoside **7** as the major product in 64% yield, presumably due to nitrilium ion coordination in the axial position.⁹ Catalytic hydrogenation of **7** over Pd/C gave the amine intermediate, which was directly transformed without further purification into the corresponding acrylamide. Finally, facile deacetylation under Zemplén conditions afforded the desired disaccharide **1** in 90% yield. Nitrophenylazide monomer **3** (Scheme S2) and arylamine initiator **4**^{6b} were readily synthesized using standard procedures.

With the monomers in hand, we generated glycopolymers **9**–**11** using acrylamide as the comonomer for the polymer backbone (Scheme 1 and Table 1). Treatment of **4** with HBF_4

Scheme 1. Synthesis of Biotin-Functionalized, Photoactivatable Glycopolymers



and NaNO_2 in degassed 1:1 $\text{H}_2\text{O}/\text{THF}$ gave the arenediazonium cation, which upon reaction with NaOCN at 60 °C generated the biotinyl aryl radical *in situ* to serve as the initiator. Addition of disaccharide monomer **1** and acrylamide (1:4 molar ratio) generated polymer **9** with desirable sugar density and water solubility. Using the same sugar to acrylamide ratio, we also copolymerized monomer **1** with nitrophenylazide monomer **3** (1:3 molar ratio) to give polymer **10**. Furthermore, we synthesized the control polymer **11** from **2** and **3**, wherein

Table 1. Polymers Generated via OCN-Mediated Radical Polymerization

pol	monomer	$[3]_0/[1 \text{ or } 2]_0^a$	x^b	y^b	z^b	M_n^c	PDI^c
9	1	—	19	—	—	26.3	1.17
10	1 and 3	1/3	30	9	—	23.9	1.22
11	2 and 3	1/6	—	7	42	23.0	1.17

^aInitial ratio of cross-linker $[3]_0$ to ligand $[1 \text{ or } 2]_0$. ^bCross-linker and ligand content in the resulting polymer were estimated by ^1H NMR (see SI for details). ^c M_n in kDa and PDI values were determined by SEC-MALS.

each disaccharide unit was replaced by 2 equiv of 3-hydroxypropyl units (1:6 molar ratio). Characterization of the polymers by size-exclusion chromatography-multiangle light scattering (SEC-MALS) revealed narrow polydispersity index (PDI) values (~ 1.2) and number-average molecular weights (M_n) of 23–26 kDa. We also estimated an average polymer composition of up to 30 disaccharides per chain by comparing the integrated signal from the phenyl protons to that of the fucose methyl protons by ^1H NMR (see SI for details). Importantly, the relative ratios of ligand (**1** or **2**) to cross-linking agent (**3**) could be tuned to reproducibly control the glycan/cross-linker content of the polymer.

We first validated our strategy using the well-established plant lectin *Ulex europaeus* agglutinin I (UEAI), which binds with weak affinity to the $\text{Fuca}(1-2)\text{Gal}$ epitope ($\text{IC}_{50} = 0.73 \text{ mM}$).¹⁰ UEAI conjugated to fluorescein was incubated with polymer **10** or **11** for 3 h at 37 °C and subsequently exposed to 365 nm light for 15 min at 4 °C. The cross-linked proteins were isolated by streptavidin affinity chromatography, resolved by SDS-PAGE, and detected by in-gel fluorescence imaging. We found that polymer **10**, but not polymer **11**, captured UEAI, indicating selective recognition of the glycan moiety (Figure 2A).

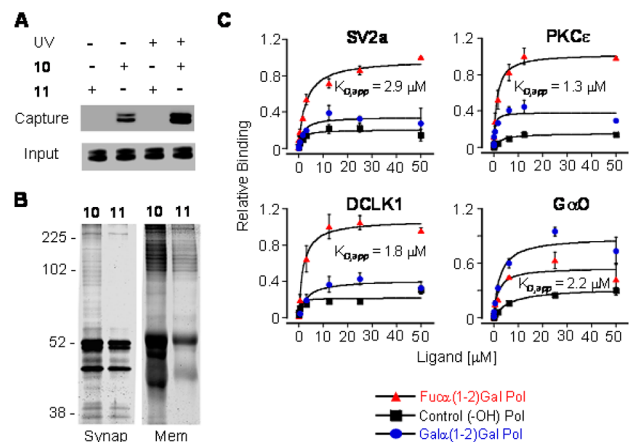


Figure 2. (A) Selective capture of UEAI by **10**. (B) Representative silver-stained gel of GBPs isolated from synaptosomal and membrane fractions. (C) Relative binding profiles of candidate GBPs ($n = 3$).

Moreover, photo-cross-linking of the polymer led to a 2-fold increase in UEAI capture. These results validate the polymer design and demonstrate that synthetic glycopolymers can be used to capture lectins with weak glycan binding affinities.

Next, we investigated whether polymer **10** could be utilized to identify novel, endogenous $\text{Fuca}(1-2)\text{Gal}$ GBPs from mammalian tissues. Various subcellular fractions from rat brain were incubated with polymer **10** or **11**, cross-linked by UV irradiation, and isolated as described above. The captured proteins were

resolved by SDS-PAGE, and the entire lanes of proteins captured either by **10** or **11** were cut into 20 similarly sized gel pieces. All gel pieces were individually subjected to in-gel proteolytic digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. We observed enhanced, specific protein capture by **10** compared to **11** in both synaptosomal and membrane fractions (Figure 2B). Individual proteins were identified using highly stringent criteria, and after filtering out any proteins present in both sample sets, 44 candidate GBPs were identified from six total experiments. Interestingly, the GBPs represented a broad range of functions, including proteins involved in signal transduction, protein trafficking, protein scaffolding, and metabolism (Table S1).

As $\text{Fuca}(1-2)\text{Gal}$ carbohydrates have been implicated in synaptic regulation and plasticity, we focused our attention on GBPs associated with those functions. Four proteins were selected for further investigation: double cortin-like kinase 1 (DCLK1), protein kinase C epsilon subunit ($\text{PKC}\epsilon$), guanine nucleotide-binding protein G(o) alpha subunit ($\text{G}\alpha\text{O}$), and synaptic vesicle glycoprotein 2a (SV2a).¹¹ Although DCLK1, $\text{PKC}\epsilon$, and $\text{G}\alpha\text{O}$ are found in the cytoplasm, they also undergo translocation to the plasma membrane.¹² Activity-dependent secretion of these proteins could provide a mechanism for interaction with extracellular $\text{Fuca}(1-2)\text{Gal}$ sugars. Indeed, secretion of cytosolic galectin 1, a lectin that binds β -galactosides, into the extracellular space has been reported to mediate T cell clearance during tumor progression.¹³ SV2a could engage $\text{Fuca}(1-2)\text{Gal}$ sugars in synaptic vesicles or at the extracellular surface after synaptic vesicle exocytosis. We assessed the binding affinities of the candidate GBPs using enzyme-linked lectin assays. Each protein was immobilized on a microtiter plate and incubated with varying concentrations of a biotinylated $\text{Fuca}(1-2)\text{Gal}$ glycopolymer. Bound polymer was detected using streptavidin conjugated to horseradish peroxidase. For comparison, we also examined the binding of a corresponding biotinylated glycopolymer bearing $\text{Gal}\alpha(1-2)\text{Gal}$ epitopes. All four proteins interacted with the $\text{Fuca}(1-2)\text{Gal}$ glycopolymer in a dose-dependent manner, albeit with different affinities (Figure 2C). Furthermore, SV2a, $\text{PKC}\epsilon$, and DCLK1 showed greater (2.5–2.8-fold) binding to $\text{Fuca}(1-2)\text{Gal}$ compared to $\text{Gal}\alpha(1-2)\text{Gal}$ glycopolymers, suggesting that the terminal fucose residue was important for recognition. In contrast, $\text{G}\alpha\text{O}$ exhibited similar binding affinity toward both $\text{Fuca}(1-2)\text{Gal}$ and $\text{Gal}\alpha(1-2)\text{Gal}$ glycopolymers ($K_{D,\text{app}} = 2.2$ and $2.1 \mu\text{M}$, respectively). Together, these results demonstrate that all four candidate GBPs recognize $\text{Fuca}(1-2)\text{Gal}$ sugars and that our glycopolymer-based approach can successfully identify novel GBPs.

As the mere presence of a glycan-protein interaction is not sufficient to establish biological function, elucidating the physiological roles of GBPs remains a major challenge. To assess whether the interaction of $\text{Fuca}(1-2)\text{Gal}$ sugars with GBPs might have important functional consequences, we studied SV2a. SV2a is a 12-transmembrane glycoprotein that has no close homology to any known animal lectins. The trafficking of SV2a between secretory vesicles and synapses is critical for activity-dependent neurotransmission.^{11c,d} To investigate the role of $\text{Fuca}(1-2)\text{Gal}$ sugars in SV2a trafficking, we utilized genetically altered mice lacking *FUT1* and *FUT2*, the fucosyltransferase genes responsible for $\text{Fuca}(1-2)\text{Gal}$ biosynthesis.¹⁴ We examined whether expression of $\text{Fuca}(1-2)\text{Gal}$ sugars was regulated by *FUT1* or *FUT2* in the mouse cortex using our recently reported chemoenzymatic strategy.¹⁵ This approach exploits an exogenous bacterial glycosyltransferase BgtA to transfer a non-

natural *N*-azidoacetylgalactosamine (GalNAz) moiety onto the C-3 position of galactose in $\text{Fuca}(1-2)\text{Gal}$ glycans (Figure S1). Subsequent bioorthogonal reaction with alkyne-functionalized fluorophores or biotin enables rapid, sensitive detection of the labeled $\text{Fuca}(1-2)\text{Gal}$ glycans. We observed significantly reduced expression of $\text{Fuca}(1-2)\text{Gal}$ glycans on cortical glycoproteins from *FUT2* knockout mice (*FUT2*^{-/-}) compared to WT littermate controls (Figures 3A and S2). In contrast, *FUT1*

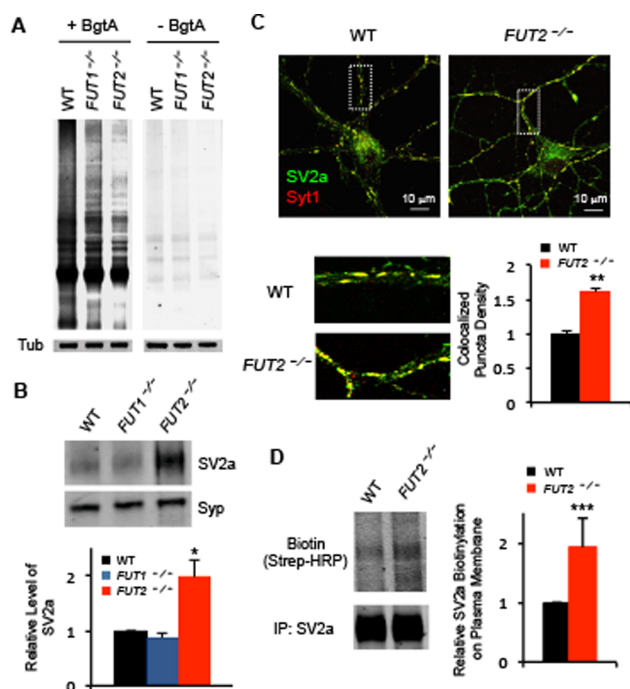


Figure 3. (A) $\text{Fuca}(1-2)\text{Gal}$ expression on glycoproteins in *FUT1*^{-/-} and *FUT2*^{-/-} mice. (B) Enrichment of SV2a in the membrane fraction of *FUT2*^{-/-} mice ($n = 3$, $*P < 0.02$). (C) Increased colocalization (yellow) of SV2a and syt1 puncta along neuronal processes in *FUT2*^{-/-} cortical neurons ($n = 70$ – 80 , $**P < 0.005$). (D) Increased cell surface biotinylation of SV2a in *FUT2*^{-/-} cortical neurons ($n = 4$, $***P < 0.05$). See SI for experimental details.

knockout mice (*FUT1*^{-/-}) showed a lower reduction in the overall levels of $\text{Fuca}(1-2)\text{Gal}$ protein fucosylation. These results suggest that *FUT2* is primarily responsible for $\text{Fuca}(1-2)\text{Gal}$ biosynthesis in the mouse cortex.

Trafficking of SV2a to the membrane influences synaptic vesicle exocytosis, also known as neurotransmitter release. Following neurotransmitter release, the endocytic reuptake of SV2a from the membrane into synaptic vesicles is critical for the proper regeneration of synaptic vesicles. To investigate whether $\text{Fuca}(1-2)\text{Gal}$ glycans influence SV2a trafficking, we examined the distribution of SV2a at the membrane in cultured cortical neurons from WT, *FUT1*^{-/-}, or *FUT2*^{-/-} mice. Notably, we found that *FUT2*^{-/-} cortical neurons exhibited a 2-fold increase in membrane-associated SV2a compared to WT or *FUT1*^{-/-} cortical neurons, as determined by subcellular fractionation and quantitative Western blot analysis (Figure 3B). In contrast, the levels of synaptophysin (syp), another synaptic vesicle protein, remained unchanged.

SV2a also regulates expression and endocytosis of synaptotagmin (syt1), a calcium sensor protein required for vesicle fusion during neurotransmitter release.^{11d} We next assessed the effect of $\text{Fuca}(1-2)\text{Gal}$ glycan loss on SV2a-mediated

endocytosis of syt1 by measuring the colocalization of SV2a and syt1 puncta at synapses in neurons from WT and *FUT2*^{-/-} mice. The density of colocalized SV2a and syt1 puncta increased by 1.6-fold in *FUT2*^{-/-} neurons compared to WT neurons, consistent with reduced endocytic reuptake (Figures 3C and S3).

Finally, analysis of SV2a trafficking requires measurement of the SV2a subpopulation exposed to the cell surface during synaptic vesicle cycling. We therefore conducted a cell-surface biotinylation assay, in which living neurons were incubated with a non-cell permeable, reactive biotin molecule to label surface proteins. Following immunoprecipitation of SV2a, we analyzed the immunoprecipitated protein by both streptavidin and Western blotting to measure the amount of SV2a on the neuronal surface compared to the total amount of SV2a precipitated. We found that the level of SV2a on the cell surface was 1.9-fold greater in *FUT2*^{-/-} neurons compared to WT neurons (Figure 3D). Thus, we show using three independent methods that SV2a localization at the membrane is disrupted in *FUT2* knockout mice, strongly suggesting that *Fuca*(1-2)Gal sugars influence SV2a function. Based on these results, we propose that the interaction of SV2a with a *Fuca*(1-2)Gal glycoprotein on the cell surface influences the endocytic reuptake of SV2a into synaptic vesicles. As *FUT2*^{-/-} mice have reduced *Fuca*(1-2)Gal sugars on their glycoproteins, this interaction is disrupted in *FUT2*^{-/-} neurons, attenuating the reuptake of SV2a into vesicles and increasing the localization of SV2a at the cell surface. As such, *Fuca*(1-2)Gal glycans may serve as a sorting signal for SV2a endocytosis during synaptic vesicle recycling. Interestingly, while SV2a itself is a glycoprotein, we found that SV2a is unlikely to be modified by *Fuca*(1-2)Gal glycans, as assessed by blotting of rat brain SV2a with UEAI (Figure S4). Together with the *in vitro* binding data, our results provide support for an important functional interaction between SV2a and *Fuca*(1-2)Gal sugars.

In summary, we have designed and synthesized glycopolymers as chemical tools for the identification of novel GBPs. These studies represent, to our knowledge, the first application of synthetic glycopolymers for the systems-level, proteomic profiling of GBPs. Notably, our polymer design enables the enrichment of endogenous GBPs that would be otherwise difficult to identify. Indeed, the present studies represent the culmination of a challenging, 12 year effort to identify *Fuca*(1-2)Gal GBPs. Using this approach, we discovered the first mammalian GBPs that recognize *Fuca*(1-2)Gal-containing glycans, and we demonstrate that the interaction of SV2a with *Fuca*(1-2)Gal sugars may have important functional consequences for SV2a trafficking and synaptic vesicle recycling. Future studies with the GBPs identified herein will continue to provide important insights into the molecular mechanisms underlying these important sugars. Moreover, we anticipate that this approach can be readily extended to discover novel GBPs for many different glycan classes.

■ ASSOCIATED CONTENT

📄 Supporting Information

Experimental details and data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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