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Deciphering the Roles of N–Glycans on Collagen–Platelet Interactions

Christian Toonstra, Yingwei Hu, and Hui Zhang*

Department of Pathology, Johns Hopkins School of Medicine, 400 N Broadway, Baltimore, Maryland 21287, United States

Abstract

Collagen is a potent agonist for platelet activation, presenting itself as a key contributor to coagulation via interactions with platelet glycoproteins. The fine details dictating platelet–collagen interactions are poorly understood. In particular, glycosylation could be a key determinant in the platelet–collagen interaction. Here, we report an affinity purification coupled to a mass spectrometry-based approach to elucidate the function of N-glycans in dictating platelet–collagen interactions. By integrative proteomic and glycoproteomic analysis of collagen–platelet interactive proteins with N-glycan manipulation, we demonstrate that the interaction of platelet adhesive receptors with collagen is highly N-glycan regulated, with glycans on many receptors playing positive roles in collagen binding, with glycans on other platelet glycoproteins exhibiting inhibitory roles on the binding to collagen. Our results significantly enhance our understanding of the details of glycans influencing the platelet–collagen interaction.

Graphical Abstract

*Corresponding Author: Tel.: (410) 502-8149. Fax: (443) 287-6388. huizhang@jhu.edu.

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Supporting Information

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1. INTRODUCTION

Platelets are crucial mediators of primary hemostasis and endothelial repair; however, chronic pathological platelet activation also plays a critical role in progressive vascular occlusion diseases.¹ Blood vessel damage exposes collagen within the subendothelial surface, which serves as the primary target site of platelet action.¹ Contact between platelet receptor proteins and nascent exposed collagen instigates platelet activation and initiates the coagulation cascade. The coagulation cascade is well-defined; however, a detailed understanding of fine details dictating the platelet–collagen interaction remains elusive. Post-translational modifications, including protein glycosylation, are potential key regulators of protein–protein interactions and have been shown to contribute to platelet protein adhesion to collagen.^{2,3} The majority of critical collagen binding platelet membrane proteins, including GPIb α , GPIIb/IIIa, CD36, GPVI, and GPIX, are glycoproteins. Previous studies have reported the significant impact glycosylation on platelet–collagen adhesion for both N-glycosylation (glycoprotein VI (GPVI), integrin β 1 (ITGB1))⁴⁵ and O-glycosylation (glycoprotein Ib α (GPIBA), integrin α Ib, integrin α 5 (ITGA5), and GPVI).^{6–8} In addition to other mechanisms, N- and O-glycosylation have been shown to play critical roles in many facets of the hemostatic system, including expression, liver clearance,^{9–14} catalytic activity,¹⁵ and signal transduction,¹⁶ as well as the proper function of the von Willebrand factor (VWF) in the clotting process.^{15,17–21}

However, there is a conspicuous dearth of information regarding the specific role of N-glycosylation dictating the binding of platelet proteins to collagen. Studies that have analyzed the impact of glycosylation on particular platelet protein function have relied predominantly on recombinant techniques for protein expression. The glycosylation of recombinant proteins varies widely based on cell type that is used to generate the proteins and thereby limits the inferences that can be made regarding the impact of native glycosylation on the platelet protein–collagen interaction.

Here, we examined the roles of N-glycans on platelet protein–collagen adhesion. In this study, we developed a method to probe the specific impact of glycosylation on protein–protein interactions (PPIs) based on the interaction of immobilized “bait” with soluble “prey” glycoproteins with and without N-glycans. The approach was validated using a simple one-to-one lectin-glycoprotein proof-of-concept system and then applied to a complex collagen (bait)-platelet protein (prey) system to elucidate the roles of glycosylation in thrombus formation. This study provides insight into the mode of binding between platelet proteins and collagen.

2. EXPERIMENTAL PROCEDURES

Platelet concentrates were obtained from ZenBio (Research Triangle Park, NC, USA). Collagen I was purchased from ChronoLog (Havertown, PA, USA). Amine-reactive resin (AminoLink) was purchased from Thermo Scientific (Rockville, IL, USA). Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Platelet Lysate Preparation

Fresh platelet rich plasma (PRP) in acid citrate dextrose buffer was diluted 1:1 (v/v) in HEPES buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 5 mM EGTA, pH 7.4) containing prostaglandin E1 (PGE1; final concentration 1 μ M). Following gentle mixing, residual white and red blood cells were removed by centrifugation at 100g for 15 min. The pellet was discarded and platelets in the supernatant were isolated from plasma via centrifugation at 800g for 15 min. The platelet pellet was washed twice with citrate dextrose saline buffer containing 10 mM sodium citrate, 150 mM sodium chloride, 1 mM EDTA, and 1% (w/v) dextrose (pH 7.4). Sedimented platelets were resuspended in an ice-cold 1:1 (v/v) mixture of platelet lysis buffer containing 0.5% Triton X-100, 30 mM HEPES, 150 mM NaCl, 2 mM EDTA, (pH 7.4), and Tyrode's buffer. The lysis buffer also contained 2 μ g/mL of aprotinin, 10 μ g/mL of leupeptin, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitor cocktail 2 (PIC2), phosphatase inhibitor cocktail 3 (PIC3), and 20 μ M O-(2-acetamido-2-deoxy-D-glucopyranosylidenamino) N-phenylcarbamate (PUGNAc). Disruption of the platelet membrane was achieved using ultrasonication in 15 s pulses (6 $\times$) at 70% maximum power. The lysed platelets were cooled on ice for 1 min between sonication cycles. Platelet lysate was separated from cellular debris by centrifugation at 1500g. The protein concentration of the platelet lysate was estimated using a bicinchoninic acid (BCA) assay (Thermo Scientific).

Collagen Affinity Column Preparation

Protein-derivatized agarose was generated using a modified version of a method reported previously by our group.⁵⁶ Briefly, 500 μL of AminoLink Plus coupling resin (1 mL of a 50% slurry) was incubated with 5-fold diluted collagen (100 μg protein in 500 μL buffer (40 mM sodium citrate and 20 mM sodium carbonate, pH 10) at 37 °C for 4 h on a rotisserie shaker. Then, 500 mM sodium cyanoborohydride in 1 $\times$ PBS was added (final concentration, 45.5 mM), and the sample was incubated at 37 °C for 2 h with mixing. The resin was washed twice with 1 $\times$ PBS and incubated with 50 mM sodium cyanoborohydride, and the sample was incubated again for 2 h at 37 °C with mixing. The unreacted aldehyde sites remaining on the beads were blocked via incubation with 1 M Tris-HCl (pH 7.6) in the presence of 50 mM sodium cyanoborohydride. The sample was incubated at 37 °C for 1 h with mixing. The resin was washed 3 $\times$ using 1 M NaCl and 10 $\times$ with 1 $\times$ PBS. The columns were stored in 1 $\times$ PBS containing 0.02% NaN_3 at 4 °C before use. Coupling efficiency was determined by BCA assay comparing the flow-through to starting solution. For both collagen I and collagen IV, coupling was >99% with nearly 100 μg of collagen loaded in each column.

Platelet Protein Collagen-Affinity Enrichment

Platelet lysate (1 mg) in PBS containing 50 mM MnCl_2 was incubated in each collagen-affinity column for 4 h at 37 °C on a rotisserie shaker. Unbound proteins were removed by sequential washes (10 $\times$ using 1 $\times$ PBS). Bound proteins were eluted with 1% trifluoroacetic acid (TFA). The eluent was neutralized with 2 M ammonium bicarbonate and dried via speedvac. Proteins in the eluent were denatured at 37 °C using 8 M urea in 1 M ammonium bicarbonate, and the disulfide bonds were reduced with 5 mM dithiothreitol (DTT). Reduced cysteine residues were alkylated at 37 °C by the addition of 10 mM iodoacetamide in the dark. The denatured, alkylated proteins were digested by the addition of a Lys-C/trypsin mix (Promega) each in a 1:50 enzyme/protein w/w ratio. The sample was incubated at 37 °C for 1 h, and the solution was diluted 2-fold with 50 mM ammonium bicarbonate to reduce the concentration of urea to 4 M. Trypsin was added in a 1:30 enzyme/protein w/w ratio and the solution incubated for an additional 1 h. The solution was diluted 4-fold to a final urea concentration of 2 M, and an additional aliquot of trypsin was added. The solution was incubated at 37 °C overnight. Following digestion, the sample was dried and dissolved in 1% TFA. The pH of the solution was acidified to pH < 2 with 10% TFA, and the tryptic digest was desalted using C18 stage tips (3M Empore, Minneapolis, MN, USA). Residual detergent was removed from label-free samples using strong-cation exchange (SCX) tips (Glygen Corp., Columbia, MD). Peptides were labeled with 10-plex Tandem mass tag (TMT) reagents (Thermo Scientific) as described elsewhere.^{57,58} TMT reagents were prepared by the addition of 40 μL of dry acetonitrile to each channel (final concentration 20 $\mu\text{g}/\mu\text{L}$). Dried peptides (5 μg) were dissolved in 200 mM HEPES, at pH 8.5, and 5 μL of the appropriate TMT reagent was added to each sample. TMT channels 126 and 127N were reserved for “blank” channels representing the background noise arising from nonspecific protein interactions with the blocked agarose beads. The remaining reagents were used to label samples in the order described in Figure S3. Labeling was performed at RT for 2 h, and the reaction was quenched by the addition of 5% hydroxylamine and shaking for 30 min. Labeled samples were acidified by the addition of 1% TFA and pooled. The pooled samples

were further acidified to pH > 2 by the addition of 10% TFA, and the pooled samples were desalted using C18 stage tips. Residual detergent was removed using SCX purification.

Liquid-Chromatography Electrospray Ionization Tandem Mass Spectrometry

Liquid-chromatography mass spectrometry (LC-MS) experiments were performed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a Dionex Ultimate 3000 RSLC nano system with a 75 $\mu\text{m} \times 15$ cm Acclaim PepMap100 separating column protected with a 2 cm guard column (Thermo Scientific). The mobile phase consisted of 0.1% formic acid in water for solvent A and 0.1% formic acid in 95% aqueous acetonitrile for solvent B. The flow rate was 300 nL/min. TMT-labeled peptides were analyzed with an Orbitrap Fusion instrument (Thermo Scientific). Intact glycopeptide analysis was performed using either the Orbitrap Fusion or Orbitrap Lumos (Thermo Scientific) equipped with an EasyLC solvent pump. The gradient profile consisting of a stepwise gradient began with 4–7% B for 7 min, 7–40% B for 90 min, 40–90% B for 5 min, and 90% B for 10 min, followed by equilibration (4% B) for 10 min. MS conditions were as follows: spray voltage was set to 2.2 kV, and Orbitrap MS1 spectra ($\text{AGC } 4 \times 10^5$) were collected from 350 to 1800 m/z at an Orbitrap resolution of 120 K followed by data-dependent higher-energy collisional dissociation tandem mass spectrometry (HCD MS/MS; resolution 60 K, collisional energy 35%, activation time 0.1 ms) of the 20 most abundant ions using an isolation width of 2.0 Da. Charge states were screened to reject both singly charged and unassigned ions; only charges between 2 and 6 were enabled. A dynamic exclusion window of 15 s was used to discriminate between previously selected ions. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE repository with the data set identifier PXD010290.⁵⁹

Database Search

All TMT-labeled LC-MS/MS data were searched using Proteome Discoverer 2.2 (Thermo Scientific) searched against UniProtKB/Swiss-Prot human protein databases.

Data Processing Software

Intact glycopeptide analysis was performed using GPQuest2.0.²⁹ All Venn diagrams were generated using eulerAPE software.⁶⁰ Heat maps were generated using Gitoools 2.3.1 software suite.

Intact Glycopeptide Analysis

Raw MS files were converted to mzML format using MSConvert from Proteowizard and searched against a peptide and glycan database using GPQuest.²⁹ Oxonium ion containing spectra were extracted using HexNAc (204.087 Da) and at least one other oxonium ion (138.055, 163.061, 168.066, 274.093, 292.103, and 366.140 Da) within a 5 ppm window as the selection criteria.

3. RESULTS

Proof-of-Concept: *Sambucus nigra* Agglutinin (SNA)-Fetuin Binding

The schematic workflow to examine the functions of glycans in protein-protein interaction (FOGIPPI) using immobilized “bait”-soluble prey protein capture is described in Figure 1a. As a validation of the concept of the workflow, we speculated that the known interaction between the immobilized plant-derived *Sambucus nigra* agglutinin (SNA), a lectin derived from elderberry bark with a known affinity for sialic acids,²² and the bovine serum glycoprotein fetuin-B could provide the ideal proof-of-concept system. Fetuin-B has three N-glycosylation sites, bearing sialylated bi- and triantennary complex-type glycans.^{23–25} Loss of sialic acids completely abrogated SNA binding (Table S1, Figure 1b). In addition, we also observed the loss of binding of Beta-2-glycoprotein 1 to SNA after sialidase treatment (Figure 1c). Beta-2-glycoprotein 1 is a highly sialylated bovine serum protein that is a commonly found contaminant in fetuin preparations.

The two forms of fetuin-B/Beta-2-glycoprotein 1 (i.e., untreated (–), and α 2, 3,6,8-neuraminidase (sialidase) treated (+)) were incubated with SNA/blank resin. Bound proteins were eluted and digested, and the trypsinized peptides were labeled with 10plex TMT reagents and subsequently analyzed via LC-MS/MS. TMT analysis largely followed expectations (Figure 1b,c), with a drop in binding by both proteins following sialidase treatment, suggesting that alterations in protein–protein interactions due to sialic acid changes can be efficiently analyzed using the immobilized bait protein-soluble prey method. The method provides a degree of flexibility as the impact of both bait and prey protein glycosylation, provided that both are glycoproteins, can be manipulated by either glycosidase or glycotransferase treatment.

Determine the Impact of N-Glycosylation on Platelet-Collagen PPIs

The fine details dictating platelet receptor protein binding to collagen are critical to understanding the nature of the adhesive receptor/protein–collagen interaction, as well as the factors necessary for optimal binding. The use of quantitative proteomics provides a nonhypothesis driven characterization of PPIs between platelet proteins and collagen with altered glycosylation states. The identification of alterations in platelet protein–collagen binding following enzymatic deglycosylation will facilitate the discovery of novel, glycan-regulated protein–protein interactions with potential, downstream therapeutic applications. We applied the FOGIPPI method to platelet protein binding to collagen. Immobilized collagen resin was generated using AminoLink agarose resin. Human platelet proteins were isolated from platelet-rich plasma (PRP) and lysed in a lysis buffer containing a nonionic detergent (Triton X-100) to ensure the solubilization of the majority of membrane-bound adhesive receptors without denaturing the protein structure or protein complexes, maintaining the capacity for protein–protein interactions (PPI). Lysed platelet proteins were treated with either PBS, PNGase F, or sialidase, and each of the treated platelet proteins were incubated with agarose-bound collagen (Figure S1).

Proteins eluted under various conditions were monitored by mass spectrometry analyses (Figure S2 and Table S2) to monitor the binding selectivity. Elution of bound proteins under

acidic conditions was chosen. Acid eluted proteins were digested via trypsin, and the trypsinized platelet proteins were labeled in duplicate TMT channels. Five individual collagen binding conditions were incorporated into two TMT sets, including native collagen and native platelet proteins (–COL/–PLT) [serves as the reference channel in each TMT set], native collagen and PNGase F-treated platelet proteins (–COL/+PLT), native collagen and sialidase-treated platelet proteins (–COL/S_{PLT}), deglycosylated collagen and native platelet proteins (+COL/–PLT), and deglycosylated collagen and deglycosylated platelet proteins (+COL/+PLT). The negative control channel consisted of native platelet proteins and Tris-blocked agarose beads (–/c). The TMT labeled peptides were pooled and fractionated using basic reverse phase liquid chromatography (bRPLC). The bRPLC fractions were concatenated (Figure S3) into 24 fractions, and LC-MS/MS analysis was used to identify and quantify changes in collagen binding following glycan manipulation.

MS/MS analysis was operated in a data-independent manner, necessitating the use of technical replicates for each channel (Figure S3). Following LC-MS/MS analysis of collagen-bound platelet proteins, a total of 2393 and 2249 proteins in sets I and II, respectively, were identified (Table S3). The relative amounts of each protein prior to deglycosylation were determined using TMT channels 128N and 128C (set I) and 127C and 128N (set II; Table S3). This high protein identification number is typical of affinity enrichment followed by 2D-LC-MS/MS analysis, as proteins that bind nonspecifically to the resin or indirectly to collagen through the protein complex formed by collagen binding proteins are included in the analysis; indeed for many proteins, the TMT intensity values were detected in the control with the blank beads.²⁶ Reproducibility analyses revealed close agreement between channels (Figure S4). Proteins that interacted with collagen were stratified based on both their statistical significance and binding strength, with a minimum inclusion criterion of 2.0-fold change and P-values of less than 0.05 in platelet protein and collagen interactions. Volcano plots were generated in order to visualize the alterations in protein binding following glycan manipulation (Figure 2), yielding distinct profiles. Significant alterations in protein binding following glycan manipulation was defined as 2-fold change up or down, where the variable dot sizes are directly related to the magnitude of the fold-change for a given protein. Under such minimal inclusion criteria, 601 platelet proteins were found to have reduced binding for native collagen following PNGase F treatment of the platelet lysate, while 109 proteins were found to have reduced binding for collagen following PNGase F-treatment. Similarly, sialidase-treated platelet lysate, PNGase F-treated collagen, and PNGase F-treated collagen/platelet proteins yielded protein ratios with at least 2-fold changes (up/down) of 485/150, 209/145, and 182/179, respectively (Figure 2). Removal of N-glycans or sialic acid from platelet glycoproteins, in general, appears to largely decrease platelet protein binding to collagen (Figure 2a,b), indicating the roles for N-linked glycans or sialic acids from platelet glycoproteins in platelet protein–collagen interactions. Conversely, collagen deglycosylation generally appears to result in higher protein binding (Figure 2c,d), indicating that the glycans on collagen may play some negative role in platelet protein–collagen interactions that limits binding.

Intact Glycopeptide Analysis Identifies the Specific Glycoproteins and Their Glycan Changes Associated with the Alterations in PPI

In order to rationalize the N-glycan dependency of binding interaction between platelet adhesive receptors/proteins and collagen, we analyzed the platelet samples with and without PNGase F treatment using intact glycopeptide analysis. Intact glycopeptide analysis allowed us to site-specifically analyze individual glycans from specific glycoproteins from a complex mixture of proteins.^{27,28} The intact glycopeptide data were searched using the software package GPQuest.^{29,30} In total, 467 unique glycopeptides were identified with a 1% false discovery rate (FDR) for the nontreated platelet proteins. For the platelet proteins treated with PNGase F, 33 unique glycopeptides were identified (1% FDR). Analysis of the overlapping glycopeptides shared between platelets without and with PNGase F treatment, with a moderate number of glycopeptides (10) in common, indicate that glycopeptides from platelets were efficiently deglycosylated (Figure S5).

Major Platelet Adhesive Receptors and Proteins Require N-Glycans for Collagen Binding

We stratified the identified proteins that were significantly impacted by deglycosylation based on interaction and biological relevance. In particular, extracellular matrix (ECM)-binding proteins, glycan binding proteins, and coagulation-related proteins were extracted from the data to look in detail at the role of N-glycosylation in platelet protein– collagen interactions within the coagulation cascade. We identified 12 collagen binding proteins, six platelet glyco-protein receptors, nine integrin proteins, five fibronectin binding proteins, 11 ECM binding proteins, eight glycan-binding proteins, and 20 coagulation-related proteins. We visualized the proteins of interest via the generation of heatmaps (Figure 3a–g). Many of the proteins highlighted exhibit pronounced alterations in platelet protein and collagen interactions following deglycosylation of the N-linked glycans or sialidase-treatment on either the platelet proteins or the immobilized collagen. In particular, the major platelet adhesive receptors exhibited a strong dependence on platelet N-glycosylation, with a significant loss of binding following de-N-glycosylation (Figure 3d,f).

To further assess the role of N-glycosylation on the adhesive interaction between platelets and collagen, we looked in detail at the 18 major adhesive proteins and receptors identified in this study and compared the TMT ratios of the fully glycosylated platelet proteins versus the deglycosylated platelet proteins, finding a significant (i.e., 2-fold) loss of binding following PNGase F treatment for 16 of the 18 proteins identified (Figure 3e). The adhesive receptors/proteins can be broadly divided into two categories: the first are collagen binding adhesive receptors/proteins and the second are other ECM binding proteins. The first categories can be subdivided into two classes, including proteins that bind directly to collagen and proteins that bind indirectly to collagen via collagen-binding proteins. The second category includes proteins that bind to other constituents of the ECM beyond collagen, including podoplanin, thrombospondin, and laminin.

Among the identified adhesive receptors/proteins that bind directly to collagen, both of the major collagen binding receptors, GPVI and (ITGA2/ITGB1), were identified, and both GPVI and ITGA2 demonstrated a > 2-fold loss of binding following PNGase F treatment of the platelet lysate, while ITGB1 was more moderately impacted (Figure 3h). Neither

receptor was significantly impacted by either sialidase treatment or collagen glycosylation. The findings for GPVI were in agreement with a prior publication which found that PNGase F-treated recombinant, GPVI, had a lower affinity for collagen.³¹ Von Willebrand Factor (VWF), a central collagen binding protein, and the first adhesive protein to initiate platelet tethering to collagen³² was found to be critically dependent upon both N-glycosylation and sialylation (Figure 3a,e) but largely unaffected by collagen glycosylation. Previous reports have shown that N-glycosylation negatively impacts platelet adhesion to VWF in the absence of collagen.³³ Our data indicate a positive role for N-glycans in VWF adhesion to collagen, at least under nonshear conditions. Consistent with previous studies,^{34,35} we found fibronectin (FN1)-collagen binding was positively influenced by N-glycosylation, and binding by FN1 to collagen dropped more than 2-fold following PNGase F treatment. In agreement with previous studies,^{36,37} we found the PPI between vitronectin (VTN) and collagen to be enhanced 2.2-fold following sialidase treatment (Figure 3a).

Many of the identified platelet adhesion receptors bind indirectly to collagen through ECM binding proteins, including VWF, FN1, and VTN. One of the most important platelet receptors is the GPIIb α , GPIIb β , GPVI, and GPIX (GPIIb-V-IX) receptor complex that forms the incipient platelet tether to VWF bound to the nascently exposed collagen. GPIIb-V-IX binds to the high-affinity surface created when VWF unrolls from a globular to a fiV-IX bin conformation on the exposed collagen surface at the site of vascular injury.^{38,39} Each individual component of the GPIIb-V-IX receptor complex demonstrated a concomitant loss (i.e., > 2-fold) of binding presumably following the loss VWF binding to collagen and, with the exception of GPIIb α/β , was similarly unaffected by collagen glycosylation (Figure 3h). Similarly, FN1 plays a central role in platelet adhesion and acts as the substrate for integrins ITGA2b/ITGB3, ITGAV/ITGB3, ITGA4/ITGB1, and ITGA5/ITGB1.⁴⁰ With the exception of ITGAV and ITGB1, the remaining FN1 adhering integrins identified exhibited a 2-fold loss in binding following PNGase F treatment of the platelet lysate (Figure 3h). The FN1/VTN binding receptor integrin $\alpha_v\beta_3$ was differentially impacted by platelet glycan manipulation, with ITGAV exhibiting little change in binding, while ITGB3 binding was found to be highly dependent upon N-glycosylation, demonstrating a 3-fold drop in binding following PNGase F treatment of the platelet lysate. The finding was in agreement with previous literature.^{41–43}

A number of ECM-binding platelet adhesive receptors were identified that targeted constituents of the ECM other than collagen. In the present study, glycoprotein IV (CD36), a multifunctional receptor known to bind to thrombospondin, was identified and found to be N-glycan dependent, demonstrating a sensitivity to PNGase F treatment of the platelet lysate and collagen (Figure 3b). In addition, several receptors bind to alternative ECM proteins, such as laminins, which are concomitantly exposed with collagen during vascular injury. ITGA6/ITGB1, which is a known laminin-binding receptor, was shown to lose affinity following deglycosylation of N-glycans from the platelet proteins (Figure 3h). The glycan binding protein C-type lectin-like receptor-2 (CLEC-2), which serves a critical role in platelet activation,⁴⁴ was also found to have lower affinity for the collagen resin following PNGase F treatment of the platelet lysate. The natural agonist for CLEC-2, podoplanin, was not identified in this study; however, CLEC-2 exhibited a 2.5-fold decrease in binding following de-N-glycosylation. For the majority of platelet adhesive receptors/proteins that

demonstrated a large binding change, the *P* values were calculated and found to be statistically significant (Figure 3i).

Roles of Glycans on Platelet Protein-Collagen Binding

Following the observation that the binding of many adhesive receptors and proteins to collagen was highly dependent on N-glycosylation, we analyzed the N-glycopeptides from the platelet adhesion receptors and collagen binding proteins that demonstrated a 2-fold change (positive or negative) in collagen binding following PNGase F treatment. Using data generated from our intact glycopeptide data, we identified glycopeptides from five collagen binding proteins and nine platelet receptor proteins that exhibited reduced/enhanced collagen binding following PNGase F treatment of platelet lysate (Table 1). Many of the glycopeptides that were identified corresponded to major collagen-binding receptors and proteins. While glycosylation of the soluble adhesive proteins exhibited the expected abundance of complex and hybrid-type N-glycans (Table 1a), many of the adhesive receptors (i.e., integrins and platelet glycoproteins) were found to contain an abundance of oligomannose glycans (Table 1b). The observation was surprising, considering the low degree of oligomannose glycans commonly identified on mammalian plasma proteins.⁴⁵ However, a number of megakaryocyte receptors, including ITGB3, have been shown to bear underprocessed N-glycans even after receptor maturation.^{46,47} The abundance of oligomannose glycans on platelet receptor proteins suggests a structural rather than an electrostatic function for N-glycosylation of platelet receptor proteins, as no significant binding change was observed following sialidase treatment (with the exception of ITGA4). Indeed, an indirect structural role for N-glycosylation, rather than a direct glycan function, has been recently suggested for ITGA2b/ITGB3 and ITGAV/ITGB3 ligand binding.⁴³ As a counter example, the ECM binding protein, histidine-rich glycoprotein (HRG), demonstrated increased binding to collagen following either PNGase F or sialidase treatment (i.e., ~5.0 and ~4.0-fold, respectively). The binding behavior of HRG could be rationalized based on HRG's substrate binding mode. HRG is known to bind to a number of negatively charged substrates via a cationic histidine-rich region.⁴⁸ Using intact glycopeptide analysis of the platelet lysate, we identified 26 unique intact glycopeptides from HRG, all of which contained highly processed hybrid and complex-type glycans, containing 23% sialylation. The loss of sialic acid via either PNGase F or sialidase treatment reduces charge repulsion with the HRG binding substrate and presumably results in improved binding affinity. The only adhesive receptor that demonstrated a significant dependence upon electrostatic interactions was ITGA4, which was more significantly impacted by sialidase treatment than by treatment with PNGase F. It should be noted that sialidase treatment, unlike PNGase F, is not specific for N-glycans and could be related to changes to O-glycans as well.

4. DISCUSSION

A number of landmark studies using AP-MS have established methods to study the impact of PTMs on PPIs.^{49–55} Established AP-MS methods are hampered by their reliance on specific mAbs and/or the use of expression tags to isolate interacting species. Here, we developed a TMT-based AP-MS method to identify the specific impact of N-glycosylation on the function of platelet adhesive receptors/proteins interacting with collagen, using

human-derived, nonrecombinant platelet proteins. The described method overcomes many limitations associated with traditional AP-MS and provides unprecedented access to probe the function of N-glycans in PPIs. Our results demonstrate that quantitative interaction proteomics using a single immobilized “bait” protein and multiple soluble “prey” proteins provides a valuable nonhypothesis driven approach to analyze the N-glycan dependence of PPIs.

Platelet adhesion to collagen is a multistep process requiring the sequential interaction of multiple adhesive receptors (GPIb-IX-V, GPVI, and Integrin $\alpha_2\beta_1$) with the extracellular matrix components collagen/VWF to facilitate secure tethering of the platelet to the disrupted vascular endothelium.³² Adhesive proteins participate in dual roles in the coagulation process by both activating platelet receptors as well as bolstering the adhesions by acting as “cement.” The application of affinity purification coupled to mass spectrometry (AP-MS) to elucidate the function of glycosylation in protein–protein interactions (FOGIPPI) provides a unique and powerful method that can potentially identify glycans that regulate coagulation-related proteins.

The approach was validated using an SNA-fetuin-B proof-of-concept system demonstrating the feasibility of the immobilized “bait”-soluble “prey” concept to study the function of glycans in PPIs. Application of the method to a complex system composed of immobilized collagen and platelet lysate revealed a striking glycan dependence among primary collagen-binding proteins. Using the immobilized “bait”-soluble “prey” method, we generated the first extensive glycan-dependent interaction analysis, identifying all major primary platelet adhesive receptors/proteins and revealing that the majority, including GP6, GPIb-V-IX, and integrin $\alpha_2\beta_1$, are strongly reliant upon N-glycosylation for proper binding interactions. It is likely that N-glycosylation could impact substrate binding either by directly facilitating or interfering with protein–protein interactions or by modulating the optimal orientation of the glycoproteins to facilitate PPIs.

Despite the advantages and utility afforded by the FOGIPPI method, there are a number of limitations that should be considered. First, endemic to all AP-MS experiments is the presence of nonspecific binding proteins in addition to the proteins of interest. In our analysis, a number of nonglycosylated cytoskeletal proteins were impacted by N-glycan manipulation; however, the change in affinity was likely an artifact of the alteration of charge/hydrophobicity following deglycosylation/desialylation. The issue of copurifying nonspecific binding proteins could potentially be addressed by incorporating unspecific binders as crucial elements in the bioinformatics pathway to determine the background binding as described in this study using conjugated resin as a control; the method was described in other AP-MS studies such as the recent label-free quantitation/stable isotope labeling by amino acids in cell culture (LFQ/SILAC) AP-MS yeast protein study.²⁶ In addition, this method is limited to identifying the impact of N-glycosylation on known PPIs by analyzing the N-glycosylation changes after removing N-glycans. Novel glycan dependent PPIs cannot be confidently assessed, as the alteration in binding could arise from the generation of a non-natural binding site created by the loss of a glycan, rather than a conformational change at a biologically relevant binding site. Additionally, the use of cell lysate is an approximation of the binding interaction between *activated* platelet proteins and

collagen; however, it cannot replicate the biological context with complete fidelity. Platelet protein binding to collagen is facilitated, under shear conditions, by a cascade of signaling pathways that result in thrombus formation. Platelet receptor activation that occurs in the cellular milieu will require intact functional platelets. The use of activated platelets during *in vitro* conditions may be possible through the application of a chemical cross-linking strategy to identify changes in binding partners before and after global platelet glycan modifications. Strategies incorporating chemical cross-linking with activated platelets to better recapitulate the biological context are currently underway. Finally, the FOGIPPI method identifies both direct and indirect interactions. The identification of indirect interactions expands the utility of the method by allowing us to probe the impact of N-glycosylation on the majority of the components of the platelet adhesion process; however, it also significantly increases the complexity of the data analysis. Moreover, some receptors which bind to collagen-binding proteins may exhibit a change in binding due either to the loss of N-glycosylation, or the loss of agonist binding to collagen, or both. Determining the source of the loss/gain of affinity by indirectly binding proteins with the immobilized “bait” is difficult to determine with confidence.

N-glycosylation appears to play a major role in platelet receptor binding to collagen and other adhesive proteins. However, given the limited impact of sialidase treatment (–COL/S_{PLT}) on platelet receptor proteins, coupled with the abundance of oligomannose glycans identified during intact glycopeptide analysis, we speculate that N-glycosylation may play a largely structural role in defining the binding interactions. The glycans may be required to maintain an optimal conformation that is critical for binding.

In summary, we have developed an FOGIPPI method utilizing proteomic approach to determine the protein–protein interactions to probe the specific function of N-glycosylation in PPIs. We have applied the method to elucidate the interaction between platelet adhesive receptors/proteins and collagen, finding that the majority of glycoproteins critical for the coagulation pathway are highly N-glycan dependent for proper adhesive function. Our first systematic FOGIPPI approach provides a rich resource to study the global impact of N-glycosylation on PPIs within the context of a complex, nonrecombinant system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proof-of-concept strategy to analyze the sialic acid dependence of protein–protein interactions using a fetuin-SNA model system. (A) Schematic representing the analytical workflow. (B) N-glycan dependence of fetuin-B binding to immobilized SNA lectin analyzed via tandem mass tag (TMT) analysis, where beads represents Tris-blocked agarose resin (reference channel, 126/127N), (–) represents no treatment (127C/128N), and (+) represents sialidase treatment (129C/130N). (C) Sialic acid dependence of SNA-Beta-2-glycoprotein 1 binding using TMT analysis. Each channel was quantified as a ratio compared to the blank reference channel 126.

Figure 2.

Comparative analysis of the loss of collagen-binding affinity between platelet proteins (PLT) and immobilized collagen (COL) following subsequent PNGase F (+) or sialidase (S) treatment of either PLT or immobilized COL via TMT-labeled quantitative proteomics. Volcano plots of all quantified proteins display the relationship between statistical significance and fold-change. The log 2 fold-change (x axis) was plotted against the $-\log_{10}$ p value (y axis). Two-fold changes and a P value of less than 0.05, indicating a loss or gain of binding, occur to the right or left, respectively, of the dashed lines. (A) Binding of PNGase F-treated PLT to untreated collagen ($-\text{COL}/+\text{PLT}$). (B) Binding of sialidase-treated platelet proteins to untreated collagen ($-\text{COL}/\text{S}_{\text{PLT}}$). (C) Binding of untreated platelet proteins to PNGase F-treated collagen ($+\text{COL}/-\text{PLT}$). (D) Binding of PNGase F-treated platelet proteins to PNGase F-treated collagen ($+\text{COL}/+\text{PLT}$). The dot sizes are directly related to the magnitude of the fold-change for a given protein.

Figure 3.

Glycan dependence of platelet protein–collagen binding for coagulation-related platelet proteins. Analysis of collagen bound adhesion receptors and other coagulation-related proteins. For each protein, the average log₂ TMT ratio was calculated by dividing the untreated reference channel (–COL/–PLT) versus the PNGase F/sialidase treated channels (+/S). (A) Heat map of identified collagen-binding proteins. (B) Fibronectin-binding proteins. (C) Coagulation-related proteins. (D) Platelet glycoprotein receptors. (E) ECM-binding proteins. (F) Platelet integrins. (G) Glycan-binding proteins. (H) Fold-change (log₂ TMT ratio) of major platelet adhesive receptors/proteins indicates a strong N-glycan dependence for most proteins. (I) P values (–log₁₀) of identified major platelet adhesive receptors/proteins.

Table 1.

Tabulated Intact Glycopeptide Analysis Results: (A) Intact Glycopeptide Analysis of Collagen-Binding Proteins Revealing Primarily Complex/Hybrid-Type Glycosylation and (B) Intact Glycopeptide Analysis of Platelet Integrin and Glycoprotein Receptors Revealing an Unusually High Degree of Oligomannose Glycans Compared to Serum Proteins

A				
prote in	# glycopeptides	highest fold-change^a	% sialylation	% oligomannose
LUM	3	3.9	0	0
SPARC	10	3.1 (−COL/S _{PLT})	0	10
VWF	19	2.4	23.5	10.5
FN1	29	2.3	22.2	6.9
VTN	24	0.46 (−COL/S _{PLT})	22.7	8.3
B				
protein	# glycopeptides	highest fold-change^a	% sialylation	% oligomannose
ITGA2	44	2.3	36	43.2
ITGA2B	4	3.1	25	25
ITGA5	7	2.2	0	71.4
ITGA6	54	2.7	32.7	9.3
ITGB3	136	3	28	39.7
ITGB5	11	3.3	0	81.8
GPIba	9	3	77.8	0
GPIV	49	2.7	39.4	32.7
GPV	18	2	11.1	0

^aUnless otherwise indicated, the highest fold-change corresponded to PNGase F treatment of platelet lysate (i.e., −COL/+PLT).