The Role of Sialylated Glycans in Human Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1)-mediated Trans Homophilic Interactions and Endothelial Cell Barrier Function*

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Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1) is a major component of the endothelial cell intercellular junction. Previous studies have shown that PECAM-1 homophilic interactions, mediated by amino-terminal immunoglobulin homology domain 1, contribute to maintenance of the vascular permeability barrier and to its re-establishment following inflammatory or thrombotic insult. PECAM-1 glycans account for ∼30% of its molecular mass, and the newly solved crystal structure of human PECAM-1 immunoglobulin homology domain 1 reveals that a glycan emanating from the asparagine residue at position 25 (Asn-25) is located within the trans homophilic-binding interface, suggesting a role for an Asn-25-associated glycan in PECAM-1 homophilic interactions. In support of this possibility, unbiased molecular docking studies revealed that negatively charged α2,3 sialic acid moieties bind tightly to a groove within the PECAM-1 homophilic interface in an orientation that favors the formation of an electrostatic bridge with positively charged Lys-89, mutation of which has been shown previously to disrupt PECAM-1-mediated homophilic binding. To verify the contribution of the Asn-25 glycan to endothelial barrier function, we generated an N25Q mutant form of PECAM-1 that is not glycosylated at this position and examined its ability to contribute to vascular integrity in endothelial cell-like REN cells. Confocal microscopy showed that although N25Q PECAM-1 concentrates normally at cell-cell junctions, the ability of this mutant form of PECAM-1 to support re-establishment of a permeability barrier following disruption with thrombin was significantly compromised. Taken together, these data suggest that a sialic acid-containing glycan emanating from Asn-25 reinforces dynamic endothelial cell-cell interactions by stabilizing the PECAM-1 homophilic binding interface.

PECAM-12 (CD31) is a type I transmembrane cell adhesion and signaling receptor that is selectively expressed on the surface of hematopoietic cells and highly enriched at endothelial cell-cell junctions (reviewed in Refs. 1, 2). The extracellular region of PECAM-1 is comprised of six Ig-like domains, followed by a 19-amino acid transmembrane domain and a 117-amino acid cytoplasmic tail containing multiple phosphorylation sites that mediate interactions with cytosolic signaling molecules (3, 4). Amino-terminal Ig domains 1 and 2 of the extracellular domain mediate PECAM-1/PECAM-1 homophilic interactions (5–8), an adhesive property of the molecule that is essential for concentrating PECAM-1 at endothelial cell-cell borders (9), sensing flow (10, 11), mediating leukocyte transendothelial migration (12, 13), conferring resistance to apoptosis (14), and regulating vascular permeability (15–17).

Mutagenesis studies performed nearly 20 years ago revealed the importance of five amino acids within IgD1 for PECAM-1/PECAM-1 homophilic interactions (7), including a critical lysine residue at position 89, as a Lys-89→alanine substitution abolishes PECAM-1-mediated homophilic interactions (7), localization to cell-cell borders (9), cytoprotection against proapoptotic stimuli (18), and the ability of PECAM-1 to contribute to the vascular permeability barrier (16). The recently solved crystal structure of PECAM-1 IgD1 and IgD2 revealed homophilic binding interfaces involving additional amino acids in both IgD1 and IgD2 (8); however, somewhat surprisingly, Lys-89 was not one of them. Thus, a molecular explanation for how mutation of this residue results in loss of homophilic binding remains a mystery.

PECAM-1 is heavily glycosylated, with ∼30% of its molecular mass contributed by nine complex N-linked carbohydrate chains (3, 19), including two within IgD1 at Asn-25 and Asn-57 and one within IgD2 at Asn-124. Interestingly, although each of these asparagine residues is located within a homophilic binding interface, as demonstrated in the recent crystal structure

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2 The abbreviations used are: PECAM, platelet endothelial cell adhesion molecule; IgD, immunoglobulin homology domain; SNA, Sambucus nigra; Gal, galactose; Siglec, sialic acid-binding Ig-like lectin(s); ICAM-1, intercellular adhesion molecule 1; ECIS, electric cell substrate impedance sensing; GlcNAc, N-acetylgalactosamine; Man3, mannose-mannose-mannose; PNGase F, peptide-N-glycosidase F.
(8), the participation of their attached glycans in homophilic binding could not be determined because the IgD1-D2 constructs used for crystallization were produced in insect cells, which have simpler N-glycans consisting only of short truncated terminal mannose residues. Kitazume et al. (20) reported the presence of terminal α2,6-linked sialic acid residues on the glycans of murine endothelial PECAM-1 and found that deletion of ST6Gal-1, which encodes the β-galactosidase sialyltransferase that adds this sugar residue to the ends of glycan chains, resulted in loss of PECAM-1 from endothelial cell-cell borders. A more recent study by the same group (21) found that α2,6-sialylated oligosaccharides inhibit murine PECAM-1 homophilic adhesion, further implicating α2,6 sialic acids in PECAM-1/PECAM-1 interactions. Murine and human PECAM-1 differ in a number of important respects, the most notable relevant example being the absence of the Asn-25-linked glycan in the murine molecule; it has a glutamine at this position. Finally, a large number of studies examining the homophilic binding properties of PECAM-1 have employed a CHO cell-secreted recombinant human PECAM-1/IgG chimeric protein that binds with high affinity to human PECAM-1 (5, 6) and has been used in vivo to block ischemia/reperfusion injury (22). Because CHO cells express only α2,3-sialyltransferases, which add terminal α2,3-linked sialic acids to the terminus of glycan chains, but lack α2,6 sialyltransferases (23–25), it is likely that human versus murine PECAM-1 differ in the molecular requirements necessary for supporting PECAM-1/PECAM-1 homophilic interactions, a concept reinforced by the long-held observation that human and murine PECAM-1 cannot bind to each other (5, 6).

The purpose of this investigation, therefore, was to examine the relative ability of α2,3- versus α2,6-linked sialic acid residues to contribute to human PECAM-1 trans homophilic interactions. Given the species specificity of the glycan at Asn-25 of human IgD1, we also examined the role of this glycan in concentrating PECAM-1 at cell-cell borders and in regulating junctional integrity. Using glycan-specific recombinant PECAM-1/IgG constructs containing only α2,3 sialic acid moieties versus both α2,3 and α2,6 sialic acids, we found that the presence of α2,6 sialic acid inhibits, rather than supports, homophilic binding of human PECAM-1. Unbiased molecular docking analysis revealed that α2,6-sialylated glycan binds across the face of IgD1 in such a way as to inhibit the ability of an Asn-25-linked glycan terminating in α2,3 sialic acid to interact with Lys-89. Taken together, these data emphasize the species-specific requirements for PECAM-1 homophilic adhesion and provide a molecular explanation for the role of Lys-89 in mediating PECAM-1 homophilic interactions.

Results

α2,6-linked Sialic Acid Residues Inhibit PECAM-1-mediated Homophilic Interactions via an Intradomain Electrostatic Interaction with Lys-89—PECAM-1 is predominantly glycosylated with hybrid and complex N-glycans (19), and previous studies have implicated terminal α2,6 sialic acid residues in supporting the ability of murine PECAM-1 to form homophilic interactions. Because human PECAM-1 contains an N-glycosylation site at amino acid 25 that is not present in murine PECAM-1, and because human PECAM-1/IgG produced in CHO cells, which lack α2,6-sialytransferase activity, has been used for many years to characterize the homophilic binding properties of PECAM-1, we examined whether addition of α2,6 sialic acids to human PECAM-1/IgG might enhance its binding ability. To accomplish this, we transfected a cDNA construct encoding PECAM-1/IgG into a specialized CHO cell line that had been stably transfected with ST6Gal-1 (a generous gift from Ajit Varki, University of California, San Diego). As shown in Fig. 1A, Sambucus nigra (SNA) lectin, which binds selectively to proteins containing α2,6 sialic acids, bound to α2,6+α2,3-sialylated PECAM-1/IgG but not to the α2,3-sialylated form of PECAM-1/IgG that had been generated from wild-type CHO cells. SNA lectin also bound to PECAM-1 expressed on REN cells (data not shown). Somewhat surprisingly, the α2,6+α2,3-sialylated form of PECAM-1/IgG was completely unable to interact homophilically with WT PECAM-1-expressing REN cells (Fig. 1B) unless it was desialylated with neuraminidase (Fig. 1C). In contrast, α2,3-sialylated PECAM-1/IgG bound in a dose-dependent manner to WT PECAM-1-expressed REN cells (Fig. 1B), whereas desialylation had only a minor deleterious effect on homophilic binding (Fig. 1D). The relative binding of each of these glycoforms, at a 100 μg/ml concentration, to PECAM-1-expressed REN cells is quantified in Fig. 1E. Thus, in contrast to murine PECAM-1, α2,6-linked sialic acids significantly inhibit, rather than support, the homophilic binding of human PECAM-1, whereas α2,3-linked sialic acid-containing glycans play only a minor role in steady-state trans homophilic interactions.

To understand the mechanism by which α2,6-sialylated glycans might inhibit PECAM-1/PECAM-1 homophilic interactions, a molecular docking analysis was used to predict the binding of α2,6-sialylated lactosamine (comprised of sialic acid, galactose (Gal), and GlcNAc, coordinates obtained from PDB code 1JSI (26)) to the recently solved crystal structure of human PECAM-1 IgD1 (8). The coordinates of this ligand were energy-minimized using PRODRG2 (27), and partial charges of α2,6-sialylated lactosamine and human PECAM-1 IgD1 were generated using the Gasteiger module in AutoDockTools (28). A cubic grid encompassing the entire surface of IgD1 was used for blind docking analysis, and the ligand was allowed to interact with the entire surface. As depicted in Fig. 2A, the resulting lowest estimated free energy of binding computed using AutoDockTools revealed that the α2,6-linked sialylated lactosamine binds across the lateral face of PECAM-1 IgD1 to form an electrostatic interaction with amino acid Lys-89, with a best estimated free energy of binding of −6.62 kcal/mol. The distance from carbon 1 of the GlcNAc residue of the docked α2,6 lactosamine residue to the nitrogen atom of Asn-57 is 16.4 Å, a spacing that easily accommodates the core N-linked antenna that can span 10–17 Å (GlcNAc-GlcNAc-Man3, PDB code 1GYA (29)), shown as three green rings attached to two blue rings in Fig. 2A) emanating from Asn-57. Because the mannose core antenna is flexible, carbon 4 of the terminal mannose can be positioned next to carbon 1 of the GlcNAc residue of the docked α2,6-sialylated lactosamine. Moreover, the α2,6 lactosamine-PECAM-1 IgD1 complex was also fitted into the crystal lattice to observe the involvement of the α2,6-sialylated...
glycan in the interactions between the docked PECAM-1 IgD1 and the opposing PECAM-1 IgD1 molecule that forms trans homophilic interactions. In contrast to the distance between the nitrogen atom of Asn-57 to the GlcNAc residue of α2,6 lactosamine, that of Asn-25 residing on the trans homophilic-interacting molecule is 19 Å, which is too large to harbor the core N-glycan. Therefore, α2,6-sialylated is not likely to emanate from Asn-25 of the opposing IgD1 molecule. For this reason, Asn-57 in the α2,6 + α2,3-sialylated form of PECAM-1/ IgG might carry an inhibitory α2,6 sialic acid residue that inhibits homophilic interactions involving Lys-89. This observation is consistent with the experimental observations shown in Fig. 1.

To determine whether α2,6 + α2,3-sialylated PECAM-1/IgG is actually modified at Asn-57 by α2,6 sialic acid, the protein was digested with trypsin/Lys-C to produce a series of glycopeptides and peptides. α2,6-Sialylated glycopeptides were captured using SNA-agarose affinity chromatography, eluted, deglycosylated with PNGase F, and subjected to tandem mass spectrometry. As shown in Fig. 2B, Asn-57 is indeed α2,6 sialylated, thus explaining the inability of α2,6 + α2,3-sialylated PECAM-1/IgG to interact homophilically with PECAM-1-expressing cells.

Molecular Modeling of the N25-linked Glysacn of Human PECAM-1 Reveals That It Forms a Molecular Bridge between Two Opposing PECAM-1 Molecules Interacting in Trans—In contrast to the α2,6 + α2,3-sialylated form of PECAM-1/IgG, α2,3-sialylated PECAM-1/IgG binds well to PECAM-1 expressed on the surface of cells (Refs. 5, 6 and Fig. 1). The recently solved crystal structure of human PECAM-1 IgD1 reveals that the asparagine at position 25 is both glycosylated and present at the trans homophilic binding interface (Fig. 3A and Ref. 8). To determine whether an α2,3-linked sialic acid-containing glycan emanating from Asn-25 might contribute to the adhesive properties of human PECAM-1, we again performed unbiased molecular docking, this time analyzing the binding of α2,3-sialylated lactosamine to PECAM-1 IgD1 using the identical docking parameters of α2,6-sialylated lactosamine. As shown in Fig. 3B, α2,3-sialylated lactosamine is predicted to bind in a groove of IgD1 in such a way as to form a hydrogen bond between its carboxyl moiety and the ε-amino group of Lys-89 with an estimated free energy of −4.8 kcal/mol. Bound in this fashion, when fitted into the crystal lattice, the hemiacetal carbon atom (C1) of the GlcNAc residue of the α2,3-sialylated lactosamine is 11.3 Å away from the nitrogen atom of Asn-25 residing on the trans homophilic-interacting PECAM-1 IgD1, providing favorable space for the core N-glycan to occupy (three green rings attached to two blue rings in Fig. 3B). This observation is consistent with the notion that α2,3-sialylated lactosamine extending from the core glycan of Asn-25 is capable of forming an interdomain molecular bridge to the Lys-89 residue of an opposing PECAM-1 IgD1 molecule interacting in trans. These modeling data predict that an Asn-
25-associated sialylated glycan might reinforce PECAM-1-mediated homophilic interactions.

The Asn-25 Glycan Supports Functional Dynamic PECAM-1-mediated Homophilic Interactions—Sabri et al. (30) recently reported that sialic acid residues have a more pronounced effect on dynamic, rather than static, cell adhesive interactions. To determine whether the glycan attached to Asn-25 plays a functional role in PECAM-1-mediated homophilic adhesion, we generated three REN cell lines, each expressing a different form of PECAM-1: WT PECAM-1, Lys-89, a homophilically crippled...
PECAM-1, and an N25Q form of PECAM-1 that lacks its associated glycan residue. As shown in Fig. 4A, the N25Q mutant form of PECAM-1 immunoprecipitated from detergent-solubilized REN cells ran with a slightly smaller apparent molecular weight, consistent with its loss of a carbohydrate chain. Although PECAM-1 IgG binding to the K89A mutant form of PECAM-1 was less than 10% of that observed for its binding to WT PECAM-1 (Fig. 4, B and C, and Ref. 7), N25Q PECAM-1 supported normal steady-state binding of α2,3-sialylated PECAM-1 IgG (Fig. 4, B and C), concentrated normally at cell-cell borders (Fig. 4D), and maintained baseline junctional integrity (Fig. 5, A and B) to the same degree as WT PECAM-1, all well described features of PECAM-1-mediated steady-state homophilic binding. In stark contrast, however, when the permeability barrier was disrupted with thrombin, the rate of recovery was severely compromised in REN cells expressing N25Q PECAM-1 (Fig. 5, A and C). These data provide strong support for the notion that the glycan attached to Asn-25 plays an important role in supporting dynamic PECAM-1/PECAM-1 homophilic interactions.

Discussion

Nearly all vertebrate cell surface receptors that pass through the endoplasmic reticulum and Golgi on their way to the plasma membrane, including those that participate in cell adhesion and signaling, are glycosylated. Although the role of glycans in cell adhesion has been best characterized in the Selectin (31) and sialic acid-binding Ig-like lectins (Siglec) (32)
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families of glycan-binding proteins, other notable cell adhesion molecules in which carbohydrate residues have been shown to play a prominent role include E-Cadherin (33, 34), neural cell adhesion molecule (35), ICAM-1 (36, 37), vascular cell adhesion molecule (38), and junctional adhesion molecule (39). Despite the fact that the molecular mass of PECAM-1 is ~30% carbohydrate (3, 19), there have been only a handful of studies examining the potential contribution of PECAM-1-linked glycans to its adhesive and signaling function, and most of these have been performed in murine cells. The purpose of this investigation, therefore, was to identify, in the context of its recently solved crystal structure, the specific glycans that emanate from the human PECAM-1 homophilic binding domain and determine how they might contribute to PECAM-1-mediated adhesion and function.

Glycans often exert their effects through the terminal sugar residue, which, in vertebrates, is typically sialic acid, a nine-carbon sugar, carbon 2 of which forms either α2,3, α2,6, or α2,8 linkages with the underlying glycoconjugate chain. α2,3 and α2,6 linkages are strongly inhibitory (Fig. 1). The expression and specific linkage of sialic acids to the underlying glycoconjugate chain is known to vary in a cell type- and vascular bed-specific manner (47) and can also be strongly influenced by the metabolic state of the cell (48) and whether the cells have been subject to various inflammatory stimuli (49–51). More important, however, is the observation that endothelial cell adhesion molecules from different species often differ in both the number and location of functional glycosylation sites. For example, human ICAM-1, vascular cell adhesion molecule, E-selectin, and P-selectin have 8, 6, 9, and 11 N-linked glycans, whereas their murine counterparts express 10, 7, 11, and 12, respectively (47). In the case of PECAM-1, murine PECAM-1 contains seven as opposed to nine N-linked glycosylation sites and is missing the major glycosylation site, Asn-25, in IgD1 that is present at the homophilic binding interface of human PECAM-1 (Fig. 3A).

A mechanistic explanation for the differential effect of α2,6-versus α2,3-linked sialic acid moieties on the ability of human PECAM-1 to interact homophobically was provided by unbiased molecular docking studies in which α2,6 sialylated and α2,3 sialylated lactosamines were allowed to interact in silico with human PECAM-1 IgD1. These studies predict that, although α2,6 lactosamine interacts with higher affinity to IgD1 than α2,3 lactosamine, in agreement with the experimental observations of Kitazume et al. (21), it binds across the face of IgD1 in such a way as to block the ability of Lys-89 to participate in homophilic binding (Fig. 2A). In contrast, molecular modeling of the binding of α2,3 sialic acid emanating from Asn-25 reveals that this glycoconjugate is capable of forming an intermolecular bridge between two opposing PECAM-1 interacting in trans (Fig. 3B), thereby reinforcing homophilic binding interactions.

A close examination of the interaction of this sialylated glycan with IgD1 of an opposing PECAM-1 molecule is shown in Fig. 6 and reveals four highly conserved amino acids, Ile-7, Asn-88, Lys-89, and Lys-91, that bind directly with the glycan. Of
any effect on human PECAM-1-mediated homophilic binding (7).

PECAM-1 has been shown in a number of laboratories to be an important contributor to the maintenance of the vascular permeability barrier as well as to its restoration following thrombotic or inflammatory insult (15, 16, 52, 53). The ability of PECAM-1 to localize to, and concentrate at, cell-cell junctions, where it carries out this function, is completely dependent on its ability to form trans PECAM-1/PECAM-1 homophilic interactions, an adhesive property of the PECAM-1 extracellular domain that was first proposed more than 25 years ago (54), shown to be due to diffusion trapping of the receptor at cell-cell junctions 10 years later (9) and most recently found to support endothelial cell junctional integrity in a potentially regulatable manner (17, 55). Interestingly, Cioffi et al. (56) have shown recently that not only are sialic acids an important determinant of endothelial barrier integrity but that, although the arterial endothelium is likely to display both α2,3 and α2,6 sialic acid residues, the cell surface receptors of microvascular endothelial cells are primarily α2,3-sialylated. Taken together with the findings presented here that α2,3-linked sialic acid residues support, whereas α2,6 sialic acids inhibit, PECAM-1-mediated homophilic interactions, it is tempting to speculate that PECAM-1 might be a target for a novel mode of regulating endothelial cell permeability; namely, dynamic glycan modification. Whether changes in the expression of glycosyltransferases and neuraminidases that take place during bouts of thrombosis and inflammation result in changes in the linkage specificity of the sialic acids bound to PECAM-1 and how this might contribute to endothelial cell junctional integrity, leukocyte trans endothelial migration, wound healing, and repair should be fascinating topics of future investigation.

**Experimental Procedures**

Construction of IgD1 N-glycan Mutants of Human PECAM-1—pcDNA3 (Invitrogen) containing wild-type PECAM-1 was used as a template to generate Asn → Gln constructs at amino acid positions 25 and 57. Site-directed mutagenesis was performed using the QuikChange Lightning kit (Agilent Technologies, Santa Clara, CA) following the instructions of the manufacturer. N25Q mutant PECAM-1 was generated using 5′-GTG CAA AAT GGG AAG CAG CTG ACC CTG CAG TGC-3′ (forward) and 5′-GCA CTG CAG GGT CAG CTG CTT CCC ATT TTG CAC-3′ (reverse) primers. The Asn-25 PECAM-1 mutant was verified by DNA sequence analysis before use.

**Cell Lines**—The human mesothelioma cell line REN (57) was maintained in RPMI 1640 supplemented with 10% FBS and 2 mM l-glutamine. REN cells are a human mesothelioma cell line that exhibits a number of endothelium-like properties, including a cobblestone-shaped, monolayer morphology with well-defined cell-cell borders and expression of numerous endothelial cell adhesion molecules (58). REN cells are also PECAM-1-negative, are easily transfectable, and have, as a result, been used for more than 20 years as endothelial cell surrogates to study PECAM-1 biology (9, 16, 59). Plasmids encoding WT PECAM-1, K89A PECAM-1, and N25Q PECAM-1 were transfected into REN cells using Lipofectamine® LTX (Thermo Fisher Scientific, Waltham, MA) following the instructions of...
Construction and Purification of PECAM-1/IgG Containing α2,3- and α2,6-linked Sialic Acid Residues—WT CHO cells express α2,3, but not α2,6, sialyltransferase activity (24). PECAM-1/IgG chimeric protein containing only α2,3-linked terminal sialic acid residues was expressed and purified from WT CHO cells as described previously (5). PECAM-1/IgG containing both α2,3- and α2,6-linked sialic acid residues was generated by transfecting PECAM-1/IgG cDNA in pcDNA3.1 into a CHO-K1 cell line that had been stably transfected with ST6Gal-1 (23), a generous gift from Dr. Ajit Varki (University of California, San Diego). Both forms of PECAM-1/IgG were purified by protein A-Sepharose chromatography, as described previously (5). Desialylated PECAM-1/IgG was prepared by incubating protein A-Sepharose-bound PECAM-1/IgG with neuraminidase (New England Biolabs, Ipswich, MA) at 37°C overnight before eluting the protein from the column.

Binding of PECAM-1/IgG to PECAM-1-transfected REN Cells—REN cells expressing wild-type and mutant PECAM-1 isoforms were incubated with varying concentrations of PECAM-1/IgG in PBS containing 10% FBS for 1 h at room temperature. Fab fragments of the mouse anti-PECAM-1 monoclonal antibody, mAb PECAM-1.2, detected with Alexa 647-conjugated goat anti-mouse IgG (H + L) (Invitrogen), were included so that PECAM-1/IgG binding could be analyzed on 647-conjugated goat anti-mouse IgG (H + L). The blots were developed with Alexa 647-conjugated goat anti-mouse IgG (H + L) (Invitrogen), which is specific for mouse IgG (Sigma) was used as a control of PECAM-1/IgG.

Immunoblotting and Lectin Blotting—PECAM-1/IgG isoforms expressing various linkages of sialic acid were subjected to SDS-PAGE and immunoblot analysis using 1 μg/ml of biotinylated wheat germ agglutinin (Vector Laboratories, Burlingame, CA), which binds to both α2,6- and α2,3-linked sialic acids as well as exposed GlcNAc residues, or SNA lectin (Vector Laboratories), which is specific for α2,6-linked sialic acid residues. The lectins were incubated with transfer membranes at room temperature for 1 h in Tris-buffered saline + Tween 20 containing 0.1 mM CaCl2, washed, and incubated with streptavidin conjugated with HRP for an additional hour. PECAM-1 antigen was detected by immunoblotting with 3 μg/ml of the anti-PECAM-1 mAb, PECAM-1.3, followed by HRP-conjugated goat anti-mouse (H + L). The blots were developed with SuperSignal™ West Pico chemiluminescent substrate (Thermo Fisher Scientific).

Identification of α2,6-sialylated Glycan Site(s) in α2,6+α2,3-sialylated PECAM-1/IgG—Lectin affinity capture was performed as described previously (60) with some modification. Briefly, α2,6+α2,3-sialylated PECAM-1/IgG was reduced with DTT (Sigma) at 56°C for 20 min and alkylated with iodoacetic acid (Sigma) at room temperature in the dark for 15 min. ProteaseMax™ surfactant (Promega, Madison, WI) was also added to facilitate denaturation of protein as described by the manufacturer. The reduced and alkylated protein was diazylated in 50 mM ammonium bicarbonate buffer overnight at 4°C to remove DTT. After that, the diazylated protein was digested with Trypsin/Lys-C mixture (Promega) following the instructions of the manufacturer. The resulting peptides and glycopeptides were subsequently incubated with SNA-agarose (Vector Laboratories) overnight at 4°C to select α2,6-sialylated glycopeptides. The SNA-bound glycopeptides were washed with 50 mM ammonium bicarbonate and eluted with 0.5 M lactose as described by the manufacturer. Then, the eluted glycopeptides were deglycosylated with PNGase F (Promega). The deglycosylated glycopeptides were desalted with ZipTip® (EMD Millipore, Billerica, MA) and subjected to LC/MS-MS. NanoFlow HPLC was performed using nanoACQUITY 10 cm × 75 μm column (Waters, Milford, MA) in-house packed with Magic C18 3-μm (New Objective, Inc., Woburn, MA). Solvent A consisted of 0.1% formic acid in deionized water, and solvent B consisted of 0.1% formic acid in acetonitrile. The effective flow rate was 300 nl/min. The gradients were as follows: solvent B, 5% 0 min, 30% 53 min, 90% 63 min, 90% 65 min, 5% 70 min, and 5% 90 min. The standard 60,000 (nominal) resolution scan on the LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) was set up in data-dependent acquisition mode to perform the survey scan. The 10 most abundant ions were selected for subsequent fragmentation using collision-induced dissociation with a relative collision energy of 35%. Orbitrap-selected precursor ions were fragmented and then sequenced in the linear ion trap. All data analyses were performed in the MaxQuant environment version 1.4.1.2 (61), and all reagents used in this section were of mass spectrometry or equivalent grade.

Immunofluorescence Staining and Confocal Imaging—REN cells were fixed with 2% paraformaldehyde for 20 min, permeabilized with ice-cold 0.5% Triton X-100 for 2 min, washed with Dulbecco’s phosphate-buffered saline containing 0.3% BSA and then stained with 10 μg/ml mAb PECAM-1.3 for 1 h before developing with Alexa 647-conjugated goat anti-mouse IgG (H + L). Cells were imaged using an FV1000-MPE laser-scanning confocal microscope (Olympus, Center Valley, PA). Cross-sectional reconstruction was done using MetaMorph software version 7.73 (Molecular Devices, Sunnyvale, CA).

Barrier Function Measurements—REN cells were plated on 8W10E+ electrode arrays precoated with 0.1% gelatin and allowed to grow to confluence for 2–3 days. Electric cell substrate impedance sensing (ECIS) measurements were performed in duplicate chambers using ECIS model Z0 (Applied Biophysics, Troy, NY) at multiple frequencies to evaluate barrier functions as described by Giaever and Keese (62) and as described previously by us (16). On the day of the experiment, the culture medium was replaced with 380 μl of RPMI supplemented with 1% FBS. Cells were allowed to achieve a stable baseline and then stimulated with 20 μl of thrombin (Sigma) at a final concentration of 5 units/ml. ECIS measurements were modeled using ECIS software as described previously (16).

Prediction of Sialic Acid Binding Modes—The recently solved crystal structure of human PECAM-1 IgD1 (8) was used in
molecular docking analysis to predict binding modes of α2,3and α2,6-sialylated lactosamine, the three-dimensional structures of which were obtained from PDBe codes 1SN and 1SI (26), respectively. The coordinates of these ligands were energy-minimized using PRODRG2 (27). Partial charges of α2,3- and α2,6-sialylated lactosamine and human PECAM-1 IgD1 were generated using the Gasteiger module in AutoDockTools (28). A cubic grid containment having 114 × 126 × 80 grid points per side with a spacing of 0.375 Å was constructed to cover the entire IgD1 for blind docking analysis, and the ligand was allowed to bind the entire surface of IgD1, where the side chains of the interacting amino acid residues are allowed to be flexible for optimization of the interactions with the ligand. Affinity maps of the grids were calculated using AutoGrid 4.2. AutoDock 4.2 (28) was employed to dock either α2,3- or α2,6-sialylated lactosamine onto IgD1 using the Lamarckian genetic algorithm, consisting of 200 runs and 270,000 generations, with a maximum number of energy evaluation set to 2.5 × 10^6. The resulting docked conformations were analyzed using AutoDockTools, and the conformation of either α2,3- or α2,6-sialylated lactosamine with the lowest estimated free energy of binding obtained from semiempirical free energy force field in AutoDock 4.2 was selected for further analysis. Graphical representation was generated using the PyMOL Molecular Graphics System version 1.2 (Schrödinger, LLC).

Statistical Analyses—Student’s t test with unequal variance was performed for all statistical analyses using Microsoft Excel and expressed as the mean ± S.D.

Author Contributions—P. L. conducted experiments, analyzed the results, and wrote the paper. C. P. developed REN cell transfection, immunofluorescence staining, and ECIS protocols. D. K. N. suggested experiments and analyzed and interpreted data. J. Z. helped design and interpret molecular modeling studies. M. J. T. designed mass spectrometry protocols and analyzed mass spectra. P. J. N. designed experiments, analyzed and interpreted data, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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