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CHAPTER THREE

Modulation of Glycan Recognition by Clustered Saccharide Patches

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Abstract

All cells in nature are covered with a dense and complex array of glycan chains. Specific recognition and binding of glycans is a critical aspect of cellular interactions, both within and between species. Glycan–protein interactions tend to be of low affinity but high specificity, typically utilizing multivalency to generate the affinity required for biologically relevant binding. This review focuses on a higher level of glycan organization, the formation of clustered saccharide patches (CSPs), which can constitute unique ligands for highly specific interactions. Due to technical challenges, this aspect of glycan recognition remains poorly understood. We present a wealth of evidence for CSPs-mediated interactions, and discuss recent advances in experimental tools that are beginning to provide new insights into the composition and organization of CSPs. The examples presented here are likely the tip of the iceberg, and much further work is needed to elucidate fully this higher level of glycan organization.

1. INTRODUCTION: GLYCOME COMPLEXITY IN NATURE

Glycans are the most diverse of the four fundamental cellular macromolecules (Marth, 2008). Glycan diversity exceeds that of DNA and RNA (with their four nucleoside constituents) and proteins (with their 20 amino acids) because the latter molecules are strictly linear. Glycans combine dozens of possible monosaccharide units into branched structures whose diversity results from linear or branched α or β linkages between monosaccharides. Thus the glycome is comprised of many unique glycans (Marth, 2008; Varki and Sharon, 2009) that can be either free (e.g., hyaluronan), or attached to proteins (glycoproteins and proteoglycans) or lipids (glycolipids). The biosynthesis of glycans and their modifications are not template-driven; rather they are the product of multiple sometimes competing, enzymatic activities. This creates vast glycan diversity. In addition, a range of glycan variations can be found on each glycosylation site, a pattern referred to as glycosylation microheterogeneity (Kornfeld and Kornfeld, 1985).

Glycans cover the surfaces of all cells in nature, forming a dense and complex layer known as the glycocalyx. Glycans are also abundant on secreted proteins and in the extracellular matrix (ECM). Every known cell is covered in glycans suggesting that the glycome is as essential to life as a genome, transcriptome, proteome, lipidome, or a metabolome (Varki, 2011). The diversity of glycan modifications generates a unique glycan "topography" for each protein, cell, and tissue. Like a forest canopy, glycome complexity

is recognizable at various levels: the identity and modification of outer or terminal monosaccharide core structure (*leaves* and *flowers*), the linkage to the underlying sugar (*stems*), the identity, and arrangement of the underlying glycans (*branches*), the structural attributes of the underlying glycans (*tree trunks*), and finally, the spatial organization of the oligosaccharides in relation to components of the intact cell surface (the *forest* itself) (Cohen and Varki, 2010). Because glycans comprise the outer face of cells, cellular interactions within and between species typically involve glycan binding and recognition. Indeed, many examples of biological recognition by glycans have been documented, typically involving glycan-binding proteins (GBPs). These interactions include antibody recognition, pathogen binding, cell adhesion, cell–cell interactions, and signaling (Ohtsubo and Marth, 2006; Sharon, 2006; Varki and Varki, 2007).

2. GLYCAN RECOGNITION BY GBPs

Glycan interactions with GBPs are often very specific, and each type of structure in the glycan forest can mediate specific interactions with certain GBPs (reviewed in Cohen and Varki, 2010). Although highly specific, these interactions often tend to be of weak affinity (K_d values in micromolar to millimolar range) (Collins and Paulson, 2004; Cummings and Esko, 2009; Varki, 1994). Glycans often achieve biologically significant binding via multivalent avidity, with their interaction involving more than one pair of partners in close physical proximity. This review focuses on GBP interactions at the highest level of glycan complexity (the *forest*), which are not well represented by current analytical techniques, even by modern glycan microarrays. At this level of glycan complexity "clustered saccharide patches" (CSPs) can be formed by multiple glycans interacting with each other, with or without involvement of proteins or lipids. The spatial organization of glycans within such patches can influence the specificity of their interaction with GBPs.

The two major groups of GBPs are lectins and sulfated glycosaminoglycan-binding proteins (Varki et al., 2009a). This review will focus on lectins, but many of the principles discussed may apply to other types of GBPs. Lectin-binding specificity is often defined by the structure of the terminal glycan, the linkage to the underlying glycans, the structure of the underlying glycans, and the type of linkage (N- or O-) to the underlying protein/lipid (Cummings and Esko, 2009; Varki, 1994; Varki et al., 2009a; Weis and Drickamer, 1996). Lectin–glycan interactions are typically

stabilized in two ways: by hydrogen bonding between amino acids in the carbohydrate-recognition domain (CRD) and the glycan hydroxyl groups, and by van der Waals packing of the hydrophobic glycan face against the aromatic amino acid side chain (Weis and Drickamer, 1996). Because these interactions tend to be weak, multivalency is often required to generate biologically relevant binding (Dam and Brewer, 2008). There are three different ways to achieve this: simple multivalency, spatial clustering of the GBPs, or clustering of the glycans into a saccharide patch.

The typical CRD of a GBP accommodates 1–4 monosaccharides (Weis and Drickamer, 1996), in some cases simple multivalency of either the GBP or the glycans is sufficient to promote binding (Fig. 3.1A). For example, the influenza hemagglutinin has a very low affinity, but high specificity for its sialylated glycan ligands. The multivalency of the hemagglutinin trimer and the large number and density of hemagglutinin molecules on the virus envelope together promote high avidity binding. In similar fashion, a

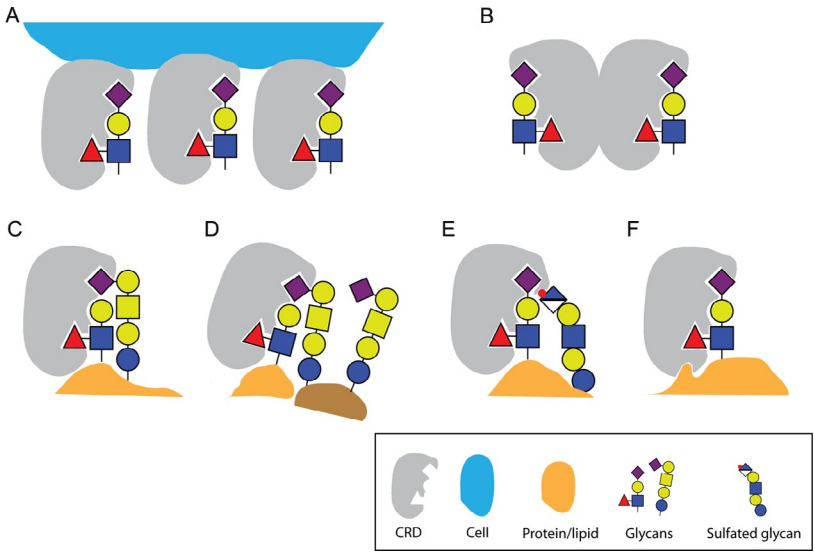

Figure 3.1 *Models of multivalent glycan binding to a carbohydrate recognition domain (CRD).* (A) Simple multivalent binding of multiple proteins to multiple glycans. (B) Multimerization of glycan-binding proteins (GBPs) can significantly enhance the affinity for glycan binding. (C–F) Binding to a clustered saccharide patch (CSP) enhances both affinity and specificity of GBP–glycan interactions. CSP may be formed by multiple glycans located on a single (C), or several (D) proteins or lipids, or by a glycan-sulfate cluster (E), or a patch comprised of glycan and adjacent peptide sequence (F).

precomplexing of recombinant soluble influenza hemagglutinins with antibodies is required for specific binding to glycans on microarrays (Stevens et al., 2006). In many cases, the high-density presentation of glycans on a microarray is sufficient to allow binding of GBP (Rillahan and Paulson, 2011). Another possible way to enhance carbohydrate–protein interactions is multivalent aggregation of the GBPs (Fig. 3.1B). For example, a pentamer of cholera toxin B subunit binds to ganglioside GM1 at a K_d value of ~40 nM (Kuziemko et al., 1996; Merritt et al., 1994). Experimental reports on avidity suggest that such multivalent interaction increase the binding affinity by a minimum of an order of magnitude compared to typical protein–carbohydrate interactions (Kuziemko et al., 1996). A third potential way to enhance both affinity and specificity of GBP–glycan interactions is by binding to a "CSP," in which several closely spaced saccharides interact to generate a specific recognition epitope (see Fig. 3.1C–F for variations in this theme) (Varki, 1994). This review focuses on this third aspect of GBP biology, which is currently poorly understood.

3. CONCEPT OF CSPs

The clustering of glycans into CSPs can be caused by multiple glycans located on a single underlying protein or lipid (Fig. 3.1C). Clusters can also involve glycans attached to two or more proteins or lipids (Fig. 3.1D). Binding patches can include glycans only, glycan-sulfates (Fig. 3.1E), or glycans and their adjacent peptide sequences (Fig. 3.1F). Within CSPs glycans are presumably presented in a distinct spatial organization that can fit the CRD pocket like a key into a lock. Since the CRD pocket can accommodate only a small number of glycans, an optimal presentation of glycans in relation to the amino acids could greatly stabilize their interactions. Saccharide clusters are stabilized by *cis* carbohydrate–carbohydrate interactions such as hydrogen bridges, hydrophobic surfaces, and ionic interactions (typically Ca^{2+} or Mg^{2+}) (Spillmann and Burger, 1996). A carbohydrate–carbohydrate interaction between glycosphingolipids also stabilizes glycosynapse microdomains (see Section 9; Hakomori, 2003). In our own work, we found that clusters of sialylated oligosaccharides and A/B blood group antigens on human erythrocytes are dispersed when a single monosaccharide is cleaved off the A/B antigen, suggesting that their spatial structure is maintained by the glycan (see Section 6; Cohen et al., 2009). The multiple lines of evidence for GBP recognition of CSPs are discussed in Sections 4–11 of this review.

Glycans are ideal molecules for mediating initial interactions since GBP–glycan interactions are highly specific but relatively weak, and thus readily reversible. Furthermore, glycan organization in CSPs can generate a unique topology even for proteins/cells with very similar glycan content (Cohen et al., 2009). Due to the complex nature of CSPs, it is extremely difficult to experimentally recreate them *in vitro* and on glycan microarrays, although several attempts have been made (see Section 11). However, there is an abundance of direct and indirect evidence for CSP-mediated binding, in particular for sialylated CSPs, which we focus on here.

Sialic acids are a family of monosaccharides typically found at the outer terminal end of glycans. They are specifically recognized by certain lectins such as Siglecs (Sialic acid-binding Ig-like lectins), and by antibodies and pathogens (Crocker et al., 2007; Varki and Angata, 2006; Varki and Varki, 2007). As with other glycans, specificity for sialic acid binding can be determined at all levels of the sialylated forest canopy (the Sialome), from modifications of the terminal monosaccharide (the *leaves* and *flowers*) to the spatial organization of the glycans (the *forest*) (Brinkman-Van der Linden and Varki, 2000; Cohen and Varki, 2010; Varki, 2008). Binding and recognition of sialic acids is known to be involved in interactions between protein–protein, protein–cell, cell–cell, cell–matrix, and is also involved in pathogen recognition.

In this review, we present examples of a spectrum of possible CSPs: on heavily glycosylated proteins (Fig. 3.2A), including CSPs formed on the cell membrane by glycosphingolipids (Fig. 3.2B) or glycoproteins (Fig. 3.2C); and CSPs on pathogens (Fig. 3.2D). All of these CSPs mediate specific interactions with certain GBPs or antibodies. We also discuss the existence of CSPs in glycosynapses, a type of glycan microdomain that mediates cell–cell and cell–ECM adhesion and signaling (Fig. 3.2E). While this review is biased toward sialylated CSPs, nonsialylated clustered patches (e.g., mannosylated clusters on HIV, Section 8.2) also exist and may be very common. Thus these examples are likely the tip of the iceberg. We expect the field to develop quickly. Recent advances in experimental tools, such as glycan arrays and lipid arrays are already providing new insight into the composition and organization of saccharides that are recognized by GBPs.

4. EVIDENCE FOR CSPs ON HEAVILY GLYCOSYLATED PROTEINS

On heavily glycosylated proteins like mucins, the polypeptide core can be completely hidden under a dense array of glycan chains. As a result, binding by other proteins is often mediated solely by the glycans, and their

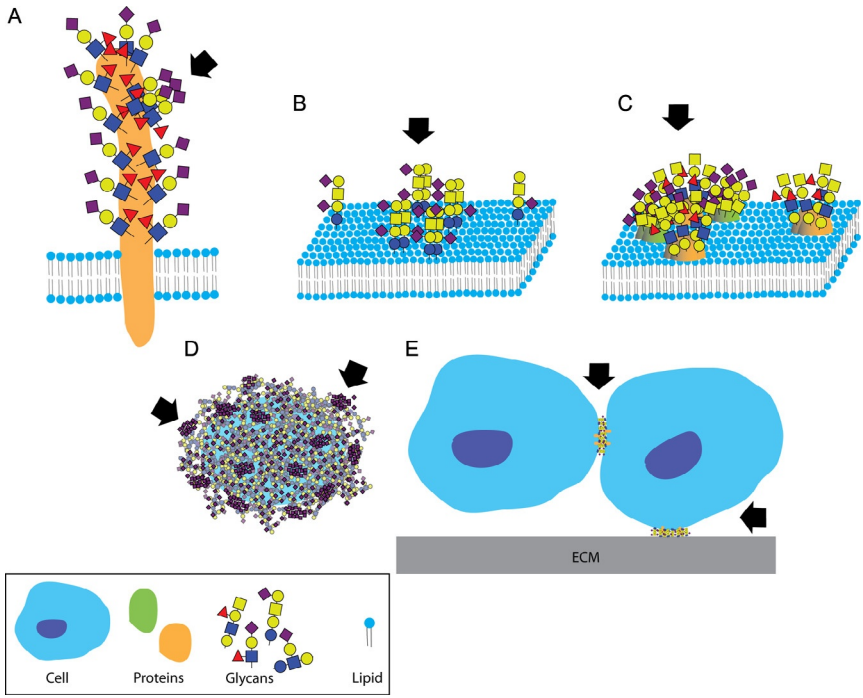

Figure 3.2 *Clustered Saccharide Patches exist at spatial scales spanning several orders of magnitude*. CSPs may form on heavily glycosylated proteins (A), on cell membranes because of interactions between two or more glycosphingolipids (B) or glycoproteins (C). CSPs can also form on pathogen polysaccharides (D). Glycosynapses are clustered glycan microdomains, which mediate cell–cell and cell–extracellular matrix adhesion and signaling (E). Arrows indicate likely CSPs.

structure and spatial arrangement can be important for recognition. These glycans are also interacting with one another in ways that restrict their motion, or cause them to cluster. Thus the complex spatial presentation of glycans in CSPs can generate novel ligands that are specifically recognized by an antibody or a receptor. We present here four examples of pathogen proteins, antibodies, and lectins that bind their targets only when glycans are clustered at high density on glycoconjugates.

4.1. Malarial merozoite recognition of clustered sialoglycans on glycophorins

Plasmodium falciparum is a major cause of human malaria. *P. falciparum* merozoites circulating in the blood invade erythrocytes via specific pathways that are either sialic acid-dependent (SAD) or -independent (SAID). The

SAD pathways are mediated by interaction between proteins from the *P. falciparum* Duffy binding-like family (e.g., pfEBA-175 and pfEBA-140), and glycophorins on erythrocytes (Oh and Chishti, 2005). Glycophorins (A, B, and C) are the major sialoglycoproteins on human erythrocytes. The extracellular domain of glycophorin A (GpA) is glycosylated with 16 O-linked glycans (primarily Neu5Acα2-3Gal(Neu5Acα2-6)GalNAcα1-*O*-Ser/Thr, with minor variations) and one sialylated N-linked glycan (Thomas and Winzler, 1969; Tomita et al., 1978). Glycophorin B (GpB) is highly homologous to GpA, but is shorter and has only 11 O-linked glycans on its extracellular domain (Anstee, 1990; Sim et al., 1994; Tomita et al., 1978). Although both GpA and GpB are modified with similar O-linked oligosaccharides, glycan distribution along the extracellular domain is unique for each protein (Anstee, 1990). Glycophorin C (GpC) is broadly similar to GpA, and contains about 13 O-linked glycans and one N-linked glycan (Anstee, 1990).

Binding of pfEBA-175 to erythrocytes is SAD, and can be inhibited by sialidase pretreatment. pfEBA-175 selectively binds to a clustered patch of six O-linked glycans on the N-terminal peptide region of a GpA dimer. However, this interaction cannot be competed by free sialic acids or by fetuin, despite the fact that the latter contains three non-adjacent O-linked glycans, which are identical to the tetrasaccharide on GpA (Orlandi et al., 1992). Furthermore, despite its high homology to GpA, soluble GpB cannot inhibit pfEBA-175 binding to erythrocytes or to GpA (Sim et al., 1994). In contrast, soluble GpA can effectively inhibit pfEBA-175 binding to erythrocytes, and inhibition with Neu5Acα2-3lactose requires a 100-fold higher concentration (Orlandi et al., 1992). pfEBA-175 binds to N-deglycosylated GpA, indicating that the sialylated N-linked glycan on GpA is not required for binding (Orlandi et al., 1992).

Taken together it is clear that the sialylated O-linked tetrasaccharide structure by itself is not the ligand for pfEBA-175, and other element(s) on GpA are required for binding. Two possible scenarios can be proposed. One is that pfEBA-175 binds to a cluster comprised of O-linked sialoglycans but also recognizes the underlying GpA peptide. The other is that GpA N-terminal peptide conformation presents the glycans in a novel clustered patch that is specifically recognized by pfEBA-175 (Sim et al., 1994). From the crystal structure of pfEBA-175 RII region (which is the erythrocyte-binding domain), it appears that the pfEBA-175 dimer binds to a clustered patch of six oligosaccharides presented on GpA (Tolia et al., 2005). Six potential glycan-binding sites were identified on the RII dimer: four are

located in the two channels of the dimer, and two are exposed to the top of the surface. Mutation analysis confirmed that all six sites are important for pfEBA-175 binding to GpA and to erythrocytes. Interestingly, several well-ordered sulfate molecules were observed in the channels and on one face of the dimer (Tolia et al., 2005). The GpA tetrasaccharide can be modeled into all six binding sites simultaneously, suggesting that pfEBA-175 ligand is a clustered patch of six *O*-glycans on the N-terminal of a GpA dimer (Tolia et al., 2005). The fact that the N-terminal peptide region of GpA and GpB is rapidly evolving among primates suggests that it is under selection pressure by the malarial pathogen (Baum et al., 2002). Consistent with this conclusion, natural human polymorphisms of this peptide sequence generate the MN blood groups (Anstee, 1990; Sadler et al., 1979). There is also evidence for rapid evolution of this peptide in five populations that have been exposed to malaria, suggesting that MN blood group polymorphism may indeed affect malarial parasite invasion (Ko et al., 2011).

There is similar evidence that another *Plasmodium* protein pfEBA-140 (BAEBL) binds to a sialylated clustered patch that includes the sialylated N-linked glycan on glycophorin C (GpC) combined with an additional unknown element. pfEBA-140 is a polymorphic Duffy binding-like receptor, each variant being identified by four amino acids in the RII region. It was determined that the BAEBL-VSTK variant recognizes GpC on erythrocytes since it does not bind to Gerbich-negative erythrocytes, which express a truncated form of GpC (Anstee, 1990; Mayer et al., 2002). The RII domain of BAEBL-VSTK has two arginine residues (Arg, R114, and R52) that are critical for binding to GpC (Jiang et al., 2009). Positively charged Arg residues are a conserved motif for sialic acid binding by Siglecs and sialidases (Varki and Angata, 2006). Erythrocyte treatment with sialidases reduces the binding of BAEBL-VSTK by 90% (Kobayashi et al., 2010), confirming sialic acid-mediated binding. Similar to pfEBA-175, fetuin does not inhibit BAEBL–VSTK interactions with erythrocytes (Kobayashi et al., 2010), despite carrying sialylated *N*-glycans with structures similar to those of GpC. In contrast to pfEBA-175, *N*-deglycosylated GpC cannot inhibit BAEBL-VSTK binding to erythrocytes, but soluble N-glycosylated GpC can. Thus the N-linked glycan is an essential component of the BAEBL-VSTK ligand on GpC. However, the isolated N-linked glycans that were cleaved from GpC cannot inhibit BAEBL-VSTK binding to erythrocytes themselves (Mayer et al., 2006). In addition, soluble GpA, which also contains a single similar N-linked glycan does not inhibit

BAEBL-VSTK binding to erythrocytes (Mayer et al., 2006) (although BAEBL-VSTK can bind to GpA on SDS-Page, Maier et al., 2003). It appears that both N-linked glycans and sialylation of GpC are required for BAEBL-VSTK binding (Malpede et al., 2013). However, the single N-linked glycan on GpC by itself is not the ligand for BAEBL-VSTK. Taken together the data suggest that similar to the pfEBA-175-GpA interaction, a cluster of N-linked glycan with other element(s) on GpC form the ligand for BAEBL-VSTK binding. The involvement of O-linked glycans in the binding was not tested, and a cluster of N-linked and O-linked glycans remains a possibility for BAEBL-VSTK binding. Alternatively, there is evidence for heparan sulfate involvement in the SAD invasion pathway of BAEBL-VSTK and GpC. Heparitinase (heparin sulfate-specific lyase) treatment inhibits merozoite invasion by a maximum of 35%, compared to almost 60% inhibition by sialidase treatment. Combined treatment with both enzymes inhibits invasion to similar extent as sialidase treatment alone, suggesting that sialic acid and heparan sulfate mediate invasion through the same pathway (Kobayashi et al., 2010). Furthermore, heparitinase treatment reduces pfEBA-140 (VSTK) binding to erythrocytes by 30–40%, and incubation with heparin (highly sulfated heparan sulfate) completely abolished binding (Kobayashi et al., 2010).

In summary, there is strong evidence that pfEBA-175 binds to a clustered patch of six *O*-glycans on the N-terminal peptide of a GpA dimer (Tolia et al., 2005). And while pfEBA-140 (BAEBL-VSTK) binding requires N-linked glycosylation on GpC, the isolated N-linked glycans are not sufficient for binding. In addition, there is evidence that heparan sulfate may be involved in the SAD pathway of BAEBL-VSTK-mediated invasion. Although erythrocytes express only small amounts of heparan sulfate (Drzeniek et al., 1999), it is possible that heparan sulfate stabilizes a sialylated cluster that is recognized by BAEBL-VSTK (Kobayashi et al., 2010; Vogt et al., 2004).

4.2. Antibody recognition of sialylated Lewis antigens on mucins

Clusters of Sialyl-Lewis^a (Siaα2-3Galβ1-3(Fucα1-4)GlcNAcβ-, SLe^a) on plasma proteins are differentially recognized by three monoclonal antibodies that were raised against SLe^a. The Lewis antigens are formed by addition of fucose to *N*-acetylglucosamine on glycolipid or glycoprotein in either α1–3 or α1–4 linkage (Stanley and Cummings, 2009). Le^a and Le^b are known as blood group antigens, as they are secreted into the plasma as glycolipids and

then incorporated into erythrocytes in individuals that are secretors. In contrast, the Le^x and Le^y antigens are expressed only on a few cell types and are not found associated with erythrocytes (Lloyd, 2000). All of the Lewis group epitopes are expressed in high densities by many carcinomas (Lloyd, 2000; Yuriev et al., 2005). Elevated levels of SLe^a bearing glycoconjugates are found in the peripheral blood of pancreatic cancer patients, and patients with acute and chronic pancreatitis, obstructive jaundice, and liver cirrhosis (Bhat et al., 2012). SLe^a is the canonical epitope for cancer/carbohydrate antigen 19-9 (CA 19-9), and is used as a biomarker for cancer diagnostics (Bhat et al., 2012; Molina et al., 2012).

Several other monoclonal antibodies are available for diagnostics: 9L426 was raised against purified SLe^a, 1B.844 antibody was raised against CA19-9 mucin antigen, and 121SLE which is comprised of mAb 19-9 and mucins that were isolated from ovarian cyst of a Lewis (a + b-) patient and further purified by immunediffusion (Gustafsson, 1990; Partyka et al., 2012). Not surprisingly, 9L426 antibody is very specific for SLe^a epitope, however both 1B.844 and 121SLE bind to additional glycan structures on glycan microarrays. For example, they both bind well to a SLe^a epitope that contains *N*-glycolylneuraminic acid (Neu5Gc), and to the SLe^c structure (SLe^a missing a fucose) (Partyka et al., 2012). Multiplexed comparison of antibodies binding to pancreatic cancer patient plasma samples revealed differential recognition of SLe^a epitopes on plasma proteins by these antibodies. The three antibodies were immobilized on microarrays, and plasma proteins from patients were incubated with these arrays. The captured proteins were then probed by each of the three antibodies, resulting in all possible antibody-pair combinations. The various capture–detection pair combinations generally showed the same pattern of recognition across the plasma samples, indicating that all three antibodies essentially recognize the same epitope (SLe^a). However, plasma proteins captured by one antibody were not always recognized by the other antibodies (Partyka et al., 2012). For example, plasma proteins from three of eleven patients were uniquely detected by 121SLE antibody but not by 9L426 and 1B.844 antibodies. In contrast, plasma proteins from two of eleven patients were recognized by 9L426 and 1B.844 antibodies but not by 121SLE (Partyka et al., 2012). This indicates that each antibody recognize a slightly different SLe^a containing epitope on the proteins. Thus the SLe^a epitopes likely form various clustered patches on the tumor-derived mucin-like proteins in plasma, which are specifically recognized by each of the antibodies. Again, we have a case where a defined glycan is necessary but not sufficient for recognition by different GBPs.

4.3. Antibody recognition of mucin-related tumor antigens Tn, TF, and STn

Incomplete mucin glycosylation in carcinomas leads to the exposure and reorganization of cryptic glycans that can be in clustered patches that are specifically recognized by certain antibodies. Examples of such cryptic glycans include the Tn (GalNAc-α1-*O*-Ser/Thr) and T (Galβ1-3GalNAc-α1-*O*-Ser/Thr) antigens, both of which have been reported in carcinomas (Springer, 1984, 1997; Varki et al., 2009b), and Sialyl-Tn (Siaα2-6GalNAc-α1-*O*-Ser/Thr, STn), which is found in some adenocarcinomas (Zhang et al., 1995).

Mucins are a family of large glycoproteins that contain variable number of tandem repeats rich in proline, threonine and serine, and are extensively O-glycosylated at the Ser/Thr residues (Itzkowitz et al., 1989; Kufe, 2009). Altered expression and incomplete glycosylation of mucins in carcinomas results in the expression of glycan epitopes that are normally cryptic. For example, mutation in the T-synthase enzyme (Cosmc) results in expression of Tn antigen, and can lead to the formation of STn (Ju et al., 2008). Reduced sialic acid O-acetylation can also expose the underlying STn structure (Itzkowitz et al., 1989; Varki et al., 2009b). Analysis of the binding pattern of two monoclonal antibodies that were raised against STn, TKH2, and B72.3 suggests that non-O-acetylated STn glycans are organized in clustered patches on colon carcinomas but not on normal tissues.

TKH2 antibody was generated following immunization with ovine submaxillary mucins (OSM) (Kjeldsen et al., 1988), which are exclusively glycosylated with STn. B72.3 antibody was generated following immunization with human breast carcinoma cells (Thor et al., 1986). Both antibodies do not bind to normal colon tissue, but interact with colon cancer tissues (Itzkowitz et al., 1989; Ogata et al., 1998). TKH2 does not bind to O-acetylated sialic acids, but de-O-acetylation of normal and cancer tissues with sodium hydroxide results in an increase in TKH2 staining (Ogata et al., 1995, 1998). This supports the hypothesis that sialic acid moieties are masked by O-acetylation in normal tissues, and de-O-acetylated in cancer tissues. In contrast, B72.3 staining was not affected by sodium hydroxide treatment (Ogata et al., 1998), however, unlike TKH2, this antibody was raised against carcinoma cells. Importantly, B72.3 strongly binds neoglycoproteins bearing STn-trimers, but poorly interacts with monomeric-STn neoglycoproteins (Ogata et al., 1998). Taken together, these findings demonstrate that in colon carcinoma tissues at least three non-O-acetylated STn epitopes form

a clustered patch that is specifically recognized by B72.3 antibody. In contrast, in normal colonic tissue STn is found in O-acetylated nonclustered potentially monomeric form (Ogata et al., 1998).

Since carcinomas express aberrant glycosylation, attempts have been made to generate anti-cancer vaccines by injecting patients with immunogenic proteins bearing the aberrant glycans. Cancer patients produce low levels of IgM antibodies against STn and Tn epitopes. These anti-STn antibodies can bind to OSM, suggesting recognition of clustered STn epitope (Adluri et al., 1995). In order to elicit production of IgG antibodies, patients were vaccinated with neoglycoproteins comprised of keyhole limpet hemocyanin (KLH) conjugated to STn (STn–KLH), or to Tn (KLH–Tn). While the vaccination enhanced the production of corresponding natural IgM antibodies, IgG antibodies from these patients reacted only with STn and Tn epitopes on synthetic conjugates (Adluri et al., 1995). The fact that IgG antibodies failed to interact with STn and Tn epitopes from natural sources such as OSM and asialoglycophorin indicates that the neoglycoproteins do not mimic the natural cancer epitope. In contrast, IgG antibodies from patients that were vaccinated with OSM reacted with both synthetic and naturally occurring STn epitopes (Adluri et al., 1995). In a separate approach, IgG monoclonal antibodies (MLS 128 and MLS 132) that were raised against human colorectal cancer cell line LS 180 were shown to specifically recognize a cluster of Tn or a cluster of STn, respectively. Immunoaffinity columns containing MLS 128 (Numata et al., 1990) or MLS 132 (Tanaka et al., 1999) antibodies were loaded with tryptic digest of OSM or asialo-OSM. All the peptides that were captured by MLS 128 contained a cluster of three or four consecutive residues of Tn antigen on a Ser-Thr-Thr glycopeptides (Nakada et al., 1991, 1993). Conversely, all the peptides captured by MLS 132 reacted with a cluster of four STn antigens on OSM peptides (Tanaka et al., 1999). Taken together it is clear that on cancer cells STn and Tn epitopes are organized in immunogenic clusters that are specifically and differentially recognized by different antibodies. As with some of the prior examples, a defined glycan is *necessary but not sufficient* for recognition by different GBPs.

4.4. Lectin binding to CA125 antigen

CA125 antigen is the most prominent marker for serous ovarian cancer; it is the extracellular domain of MUC16 mucin and is heavily O-glycosylated (Bouanene and Miled, 2010; Yin et al., 2002). It has an unusual expression of sialylated branched core 1 antennae in the core 2 glycans. Despite having a

comparable content of Sias in α2-3-linkage and α2-6-linkage, the binding pattern of recombinant soluble Siglec-Fc chimeras was found to be unique for each of three CA125 isolates. This suggests recognition by the Siglecs of sialylated CSPs that are unique for each isolate.

CA125 was isolated from three different sources: ovarian carcinoma patients (pfCA125), ovarian carcinoma cell line (clCA125), and noncancer source (pCA125). The isolates were extensively analyzed by lectin-affinity columns (Jankovic and Milutinovic, 2008), and by binding of recombinant human Siglecs to immobilized CA125 (Mitic et al., 2012). Overall the three CA125 isolates appeared to have similar sialylation patterns, as both *Maackia amurensis* lectin (MAA, recognizes Siaα2-3Galβ1-R/3-*O*-sulfate-Galβ1-R) and *Sambucus nigra* agglutinin (SNA, binds to Siaα2-6Gal/GalNAc) recognized all isolates at a similar extent (Mitic et al., 2012). Thus the proportion of Sias in α2-3- and α2-6-linkage is comparable between all three isolates. In contrast, six Fc-chimeric Siglec proteins (Siglec-2, 3, 6, 7, 9, and 10) differentially bound to the CA125 isolates. The isolate from ovarian carcinoma patients, pfCA125, was exclusively recognized by Siglec-2, 3, 6, and 7; the ovarian carcinoma cell line isolate, clCA125, was exclusively recognized by Siglec-9 and 10; and the pCA125 isolate from a noncancer source was recognized by Siglec-2, 3, 6, 9, and 10 (Mitic et al., 2012). Note that all these Siglecs can bind to various glycan structures containing Sias in α2-3- and α2-6-linkage. Specifically, Siglec-2 binds non-9-*O*-acetylated Siaα2-6Gal/GalNAc (Brinkman-Van der Linden et al., 2002; Sjoberg et al., 1994; Varki and Angata, 2006), Siglec-3 binds mostly Siaα2-6Gal and Siaα2-3Gal (Brinkman-Van der Linden and Varki, 2000), Siglec-7 preferably binds to branched α2-6-linked Sias, such as Galβ1-3(Siaα2-6)HexNAc (Angata and Varki, 2000; Yamaji et al., 2002), Siglec-6 binds Siaα2-6GalNAc (STn) (Brinkman-Van der Linden and Varki, 2000; Patel et al., 1999), Siglec-9 and Siglec-10 preferably bind to Siaα2-3Galβ1-4GlcNAc, although they bind to Siaα2-6Gal as well (Varki and Angata, 2006). All these are structures that are recognized by MAA and SNA, which bind to the three CA125 isolates in a comparable manner (Mitic et al., 2012). Thus the differential binding by Siglec-chimeras implies a higher order organization of the sialylated glycans on CA125, likely in sialylated CSPs.

5. CLUSTERED PATCHES OF SIALYLATED GANGLIOSIDES ON CELL SURFACES

Gangliosides are sialylated glycosphingolipids, composed of a ceramide lipid tail attached to an oligosaccharide chain that contains between

one to five sialic acid residues. Gangliosides typically form clusters on the plasma membrane, and are important components of the nervous system. Saccharides from two or more neighboring gangliosides can form a clustered patch that is uniquely recognized by antibodies. The formation of such CSPs can elicit novel antibodies. Alternatively, it can mask antibody recognition of a single ganglioside. Here we present examples of antibodies that specifically recognize aberrant CSPs, and show that clustered patch formation on melanoma cells can mask antibody recognition.

5.1. Autoantibody recognition of ganglioside complexes in immune neuropathies

Autoantibodies from patients can specifically recognize heterogeneous ganglioside clusters and yet do not react with the constituent gangliosides individually. Autoantibody–ganglioside interactions are a critical pathogenic factor in a subset of autoimmune neuropathies, including multifocal motor neuropathy (MMN), Guillain–Barré (GBS) and Miller-Fisher (MFS) syndromes (Willison and Yuki, 2002; Yuki and Hartung, 2012). Anti-ganglioside antibodies trigger inappropriate activation of the complement cascade, which may induce nerve injury in GBS and MFS patients (Kaida and Kusunoki, 2010; Willison et al., 2008). Serum IgG autoantibodies against GM1, GD1a, GT1a, and/or GQ1b gangliosides are associated with nerve injury in GBS and MFS patients (Greenshields et al., 2009; Willison and Yuki, 2002). IgM autoantibodies against GM1 and GM2 have been reported in the serum of MMN patients (Nobile-Orazio et al., 2010; Willison and Yuki, 2002). Because these antibodies are characteristic of the acute phase of the disease, they are useful diagnostic markers. However, acute phase sera from 8% to 17% of GBS patients does not react with purified gangliosides such as GM1, GM2, GM3, GD1a, GD1b, GD3, GalNAc-GD1a, GT1b, and GQ1b. Instead the serum IgG strongly reacted with ganglioside mixtures (Kaida et al., 2004, 2007; Kusunoki and Kaida, 2011).

Gangliosides are localized in glycosynapse microdomains (see Section 9 below), with the oligosaccharide side chain protruding from the plasma membrane. The sialylated oligosaccharide chains may form a saccharide cluster that can itself be specifically recognized by antibodies. Indeed, sera from certain neuropathy patients that do not react with an individual ganglioside do react with a complex of two different gangliosides (Kaida et al., 2004; Kanzaki et al., 2008; Mauri et al., 2012; Nobile-Orazio et al., 2010; Willison and Yuki, 2002). Conversely, antibody recognition of a single ganglioside may be masked in the ganglioside complex by the neighboring glycans (Ogawa et al., 2009). For example, a GA1/GQ1b ganglioside mixture

can be recognized by monoclonal anti-GQ1b antibody but not by monoclonal anti-GA1 antibody (Ogawa et al., 2009). Similarly, anti-GM1 IgM reactivity with GM1 is abolished or substantially reduced in GM1/GD1a ganglioside mixture (Nobile-Orazio et al., 2010).

The epitope recognized by serum antibodies was identified by extensive analysis of antibody binding to a mixture of two gangliosides. Two gangliosides were mixed and separated on thin layer chromatography (TLC) in a solvent that allowed overlapping in the same lane. Serum IgG from GBS patients reacted strongly with the overlapping portion, but did not react with the individual gangliosides (Kaida et al., 2007; Kusunoki and Kaida, 2011). Similarly, serum IgG bound strongly to wells coated with mixtures of GD1a/GD1b, GM1/GD1a, GD1b/GT1b, GM1/GT1b, or GM1/GD1b but not with the individual gangliosides in enzyme-linked immunosorbent assay (ELISA) (Kaida et al., 2004, 2007). Most of the anti-GD1a/GD1b- or anti-GM1/GD1a-positive sera also reacted with GM1/GD1b and GM1/GT1b ganglioside mixtures. This suggests that serum antibodies recognize a clustered epitope formed by a combination of Galβ1-3GalNAc (on GD1b or GM1) and Neu5Acα2-3Galβ1-3GalNAc (on the GT1b or GD1a) (Willison et al., 2008).

Serum antibodies from MFS patients can be divided into three groups: GQ1b- or GT1a-specific, GQ1b/GM1-reactive, and GQ1b/GD1a-reactive. GQ1b/GM1-reactive antibodies bind to ganglioside complexes containing a combination of Galβ1-3GalNAc and Neu5Acα2-8 Neu5Acα2-3Galβ1-3GalNAc in the terminal residue of ganglio-*N*-tetraose, including GQ1b/GD1b, GT1a/GD1b and GT1a/GM1 complexes. GQ1b/GD1a-reactive antibodies bind to complexes containing Neu5Acα2-3Galβ1-3GalNAc and Neu5Acα2-8Neu5Acα2-3Galβ1-3GalNAc, including GT1a/GD1a, GQ1b/GT1b and GT1a/GT1b (Kaida et al., 2006; Kusunoki and Kaida, 2011; Ogawa et al., 2009; Willison et al., 2008). In addition to the terminal sialic acid residues, the binding of certain antibodies may require one or two sialic acids on the internal galactose as well. For example, GQ1b/GA1 and GT1a/GA1 have the same terminal structure as the GQ1b/GM1 complex, but are missing one sialic acid on an internal galactose. GQ1b/GM1-reactive sera do not react with these complexes (Ogawa et al., 2009). IgM antibodies from sera of chronic neuropathy patients (MMN, chronic inflammatory demyelinating polyradiculoneuropathy and IgM paraproteinemic neuropathy) recognized GT1b/GM1, GT1b/GM2, and GM2/GD1b complexes (Nobile-Orazio et al., 2010). Additional evidence was obtained by glycan microarray studies

(see Section 11.1). Taken together from these examples, it is clear that gangliosides form specific sialylated CSPs that are detected by autoantibodies from neuropathy patients.

5.2. Antibody recognition of ganglioside complexes on melanoma cells

Heterogeneous ganglioside cluster formation on melanoma cells inhibits recognition of GM2, GM3, and GD3 by certain antibodies. Gangliosides are overexpressed in tumor cells and thus are putative targets for cancer vaccines (Heimburg-Molinaro et al., 2011). GM3 and GD3 are the predominant gangliosides expressed in melanoma tissues and cell lines (Lloyd et al., 1992; Tsuchida et al., 1989). Despite the uniformly high GM3 content, monoclonal antibodies against GM3 selectively interact with certain melanoma cell lines but not others (Lloyd et al., 1992; Wakabayashi et al., 1984). For example, the three anti-GM3 monoclonal antibodies, M2590 (IgM), CMR6 (IgM), and DH2 (IgG3), reacted only with one out of six melanoma cell lines tested (B78, mouse melanoma). Unlike the other five cell lines, B78 expresses only GM3. The other five cell lines express GM2, GD3 or other gangliosides in addition to GM3 (Lloyd et al., 1992). Similarly, anti-GD3 and anti-GM2 monoclonal antibodies (IgG3) reacted poorly with certain cell lines despite the high content of GD3 and GM2 in these cells. This phenomenon was observed on cells in suspension as well as in monolayers. To rule out that the lack of reactivity is due to crypticity effect (steric interference caused by neighboring glycoproteins), the cells were pretreated with trypsin-EDTA buffer or with pronase. Neither of the protease treatments significantly increased reactivity with anti-GM3 or anti-GD3 antibodies (Lloyd et al., 1992). Thus antibodies specific for GM3, GM2, and GD3 failed to recognize their ganglioside epitope when certain other gangliosides were present on the plasma membrane. This suggests the formation of a new epitope comprised of two or more gangliosides in a heterogeneous cluster. Gangliosides in the cluster are no longer exposed to antibody recognition, thus the single ganglioside is masked by formation of a CSP epitope.

Heterogenous GM1/GM3 clusters were also observed in normal mouse fibroblasts by immune-electron microscopy (Fujita et al., 2007). Antibodies against GM3 (monoclonal) and GM1 (polyclonal) detected clusters of $<$100 nm in diameter. GM3 and GM1 clusters were mostly segregated from each other, however, in 13.3% of the cases GM1 and GM3 co-clustered (Fujita et al., 2007). It is therefore likely that a CSP comprised of gangliosides is formed in these cells.

6. CLUSTERED PATCHES OF SIALYLATED GLYCOPROTEINS ON CELL SURFACES

The neutral ABH(O) blood group antigens stabilize sialylated clusters on human erythrocytes, unique for each blood type, that are differentially recognized by sialic acid-binding proteins. Erythrocytes from all three blood groups display a comparable amount of sialic acids (Bulai et al., 2003), most of which is found on glycophorins (see Section 4.1; Anstee, 1990). However, we found that three sialic acid binding proteins that are all specific for sialic acid in α2–6 linkage (and do not bind to ABH antigens) differentially interact with erythrocytes from each blood type (Cohen et al., 2009). While human Siglec-2 (CD22) binds to erythrocytes with blood group preference A > O > B, the pandemic human influenza hemagglutinin (A/South Carolina/1/18) binds equally to erythrocytes from all blood groups, and the plant lectin *Sambucus nigra* agglutinin (SNA) binding preference is A > B > O (Cohen and Varki, 2010; Cohen et al., 2009). Although a minor fraction of the glycan modifications on glycophorins contain the ABH antigens, it cannot explain the striking difference in Siglec-2 and SNA binding to erythrocytes from blood type B and O. Both lectins have comparable binding patterns in ELISA assay and on microarrays with strong preference to Siaα2-6Gal/GalNAc (Brinkman-Van der Linden et al., 2002; Padler-Karavani et al., 2012; Sjoberg et al., 1994; Varki and Angata, 2006). Although SNA binds to 9-*O*-acetylated sialic acids (Padler-Karavani et al., 2012) and Siglec-2 preferably binds to non-9-*O*-acetylated sialic acids (Brinkman-Van der Linden et al., 2002). But 9-*O*-acetylated Neu5Ac comprise less than 1.5% of the total sialic acids on erythrocytes and is comparable between the three blood groups (Bulai et al., 2003). Thus 9-O-acetylation cannot explain the differential binding of SNA and Siglec-2.

The majority of the ABH(O) blood group antigens on erythrocytes are found on the anion transport protein (band 3) and the glucose transport protein (band 4.5), which unlike glycophorins, are not much sialylated (Anstee, 1990). The blood type H antigen structure (Fucα1-2Galβ-R) is the precursor for both blood type A (GalNAcα1-3[Fucα1-2]Galβ-R) and blood type B (Galα1-3[Fucα1-2]Galβ-R) antigens (Greenwell, 1997; Hosoi, 2008). Note that these ABH(O) blood group antigens are neutral oligosaccharides that differ only in respect to a single terminal saccharide. Interestingly, blood type A and B antigens stabilize sialylated CSPs on the plasma membrane, but blood type H antigen does not. From electron microscopy analysis blood

type A antigens are organized in the periphery of sialylated clusters, and blood type B antigens are found in the center of sialylated clusters. In membranes of blood type O erythrocytes, very small sialylated clusters are formed and the blood type H antigens are not associated with them. Selective enzymatic cleavage of the terminal GalNAc and Gal from blood type A and B antigens, respectively, disperse the clusters and convert Siglec-2 and SNA binding in the expected direction (Cohen and Varki, 2010; Cohen et al., 2009). There is some evidence for co-localization of band 3 and GpA, which are the two most abundant proteins on the human erythrocyte membranes (Che and Cherry, 1995; Telen and Chasis, 1990; Young et al., 2000). However the interaction is not stable enough to be detected by standard methods such as co-immunoprecipitation, and fluorescence resonance energy transfer (Cohen et al., 2009; Jarolim et al., 1994; Telen and Chasis, 1990; Young et al., 2000), suggesting that band 3 and GpA do not form a stable protein complex. Taken together it is clear that sialylated clustered patches are formed on the plasma membrane of human erythrocytes, and are likely stabilized by carbohydrate–carbohydrate interactions with blood type A and B terminal saccharides. Lectin binding to sialylated ligands can be enhanced or reduced by the cluster formation resulting differential binding to cells presenting sialic acids with similar structure, linkage, and underlying sugars.

7. ENZYME RECOGNITION OF CLUSTERED GLYCANS: *O*-SIALOGLYCOPROTEIN ENDOPEPTIDASE

Proteolytic cleavage requires enzyme recognition of the target protein, and most endopeptidases recognize specific amino acid sequence motifs adjacent to the cleavage site (Turk et al., 2012). In contrast, *O*-sialoglycoprotein endopeptidase (OSGPase) binds to a clustered sialylated glycan patch on many different O-linked-glycan containing mucins and cleaves the adjacent peptide. This enzyme was first identified in the bacterium *Pasteurella haemolytica* (Abdullah et al., 1991; Lee et al., 1994; Mellors and Lo, 1995), but has homologs in all kingdoms of life (Nichols et al., 2006; Seki et al., 2002). The amino acid sequence of the *Pasteurella* enzyme substrate must contain at least 30% serine/threonine residues, thus presenting a mucin-like array of O-linked glycans (Mellors and Lo, 1995; Mellors and Sutherland, 1994). Terminal sialic acids on the O-linked oligosaccharides are also required for enzyme recognition, although sialic acids by themselves are not sufficient ligands for the enzyme (Abdullah et al., 1992; Sutherland et al., 1992).

OSGPase binds and cleaves only proteins with dense arrays of sialylated O-linked glycans, such as GpA (Mellors and Lo, 1995). As mentioned earlier, GpA contains 15 O-linked glycans and 1 N-linked glycan. Eleven of the O-linked glycans are found on serine/threonine residues that are clustered between amino acids 2–26 on the N-terminal domain, and the other four are located between amino acids 37–50 (Tomita et al., 1978). The major cleavage site of OSGPAse is the peptide bond at Arg-31-Asp-32 (Abdullah et al., 1992), which is adjacent to the O-glycosylation sites. Another example is CD43 (leukosialin, sialophorin): the extracellular domain of human CD43 is comprised of 234 amino acid, and 70–85 O-linked sialoglycans, an average of one O-linked sialoglycan per every three amino acids (Cyster et al., 1991). In keeping with this, OSGPase cleaves this protein to very small fragments (Sutherland et al., 1992). In contrast to the homogenous O-glycosylation of CD43, the 330–550 amino acid extracellular domain of CD45 (leukocyte common Ag) can be divided to three subdomains: an O-linked glycosylation region, and two separate cysteine-rich regions (Thomas, 1989; Tomita et al., 1978). The O-linked region spans about 177 amino acids at the very tip of the N-terminal domain, and is comprised of 34% serine/threonine residues, which are O-glycosylated. The two cysteine-rich domains are heavily N-linked glycosylated (Thomas, 1989). OSGPase digests only the O-linked glycosylation region of CD45, leaving the N-linked glycosylated region intact (Sutherland et al., 1992). Other examples of densely O-sialoglycosylated proteins that are cleaved by the enzyme are: bovine submaxillary mucins (Norgard et al., 1993a), hyaluronan receptor CD44 (Sutherland et al., 1992), CD34 (Sutherland et al., 1992), and epitectin (Hu et al., 1994).

OSGPase does not digest proteins that present sialylated O-linked glycans that are not densely packed, for example, bovine fetuin (Akai et al., 2003; Nwosu et al., 2011), or human IgA1 (Mattu et al., 1998), both of which contain only a small cluster of 3–4 O-linked sialylated glycans (Sutherland et al., 1992). The enzyme also does not digest N-linked sialoglycoproteins, over 30 of which have been tested to date (Mellors and Sutherland, 1994; Sutherland et al., 1992). When presented with an intact cell, the enzyme specifically cleaves only mucin-like sialoglycoproteins (Hu et al., 1994). Taken together it is clear that this enzyme requires a patch of sialylated O-linked glycans for binding to the target protein, and it cleaves that peptide bond adjacent to the O-glycosylated cluster. On the other hand, there is no specific sialoglycan sequence required for recognition. Thus OSGPase is apparently recognizing

some form of clustered sialylated patch that is common to all mucin-type glycoproteins, despite the different *O*-glycan structures.

8. CSPs ON PATHOGENS

8.1. Glycan clusters on bacterial capsular polysaccharides

There is evidence for antibody or Siglec recognition of saccharide clusters on bacteria capsules. Certain IgG-type monoclonal antibodies exclusively bind to intact type III group B Streptococcus (GBS III) but do not bind purified capsular polysaccharide (CPS) or comparable glycans on a microarray. Both gram-positive and gram-negative bacteria express extracellular polysaccharides that can form capsules and biofilms, both of which are major virulence factors (Bazaka et al., 2011). GBS is the major cause for pneumonia, septicemia, and meningitis in newborns (Maisey et al., 2008; Pincus et al., 1998, 2012), and has a capsular polysaccharide (CPS) that forms the outermost layer of the bacteria. The CPS helps bacteria to evade the host immune system by masking antigenic determinants, mimicking host glycans or by interfering with complement-mediated killing (Cieslewicz et al., 2005; Nizet and Esko, 2009). The GBS CPS is typically comprised of repeating monosaccharide units that are joined by glycosidic linkages to serotype-specific configuration (Bazaka et al., 2011; Cieslewicz et al., 2005). The CPS of GBS is comprised of 3–4 monosaccharide units: glucose (Glc), galactose (Gal), and *N*-acetylneuraminic acid (Neu5Ac) that may be O-acetylated, and in some serotypes *N*-acetylglucosamine (GlcNAc) is also present. These monosaccharides are polymerized in nine different configurations, unique for each serotype (Cieslewicz et al., 2005; Lewis et al., 2004). For example, the capsular polysaccharide of GBS III (CPSIII) is comprised of the repeating subunit Glcβ1-6 (Neu5Acα2-3Galβ1-4)GlcNAcβ1-3Gal (Wessels et al., 1987).

Three IgG-type monoclonal antibodies derived by immunization with intact CPSIII conjugated to tetanus toxoid: S3.1A6 (IgG1), S3.2A6 (IgG1), and S3.1B1 (IgG2a) (Zou et al., 1999), can bind and opsonize live GBS *in vitro* (Pincus et al., 2012). Immunization with tetanus toxoid conjugates typically yields antibodies that primarily recognize conformational structures (Kasper et al., 1996; Marques et al., 1994). None of the three antibodies bind to sialoglycans on the glycoarrays, including oligosaccharide structures that were recognized by other GBS type-III-specific antibodies (Pincus et al., 2012). S3.1B1 antibody binds equally well to sialidase-treated or untreated type III GBS in ELISA assay, suggesting that the sialic acid Neu5Ac is not part of the epitope recognized by this antibody (Pincus

et al., 2012). However, S3.1B1 antibody does not bind to *S. pneumoniae* type 14, which has the same core structure as desialylated CPSIII (Zou et al., 1999). This could be due to incomplete digestion by sialidase, or it may indicate that S3.1B1 ligand is stabilized by additional structure on CPSIII (Pincus et al., 2012). Despite the fact that the antibodies were derived by immunization with CPSIII, S3.1A6, and S3.2A6 preferably bind intact type III GBS over purified CPSIII in ELISA assay. These two antibodies do not bind desialylated GBS, indicating that Neu5Ac is a critical component of their ligand. Similarly, monoclonal antibody SS8 (IgG2a) that was derived by immunization with heat-killed type III GBS bound to intact type III GBS bacteria but not to CPSIII (Egan et al., 1983), suggesting that spatial organization of the glycans comprising SS8 ligand on intact bacteria is critical for the antibody recognition. Although the glycan components remain the same in purified CPSIII, the spatial organization is not preserved and the ligand is apparently scrambled, preventing antibody recognition (Pincus et al., 2012).

In addition to the cluster-specific IgG-type antibodies mentioned here, IgM-type antibodies preferably bound intact type III GBS over CPSIII. These antibodies were elicited by immunization with head-killed GBS (Egan et al., 1983; Pincus et al., 1998). However, unlike the IgG antibodies, the IgM antibodies bound to relevant structures on glycan microarray, with the exception of one antibody (SB3). In contrast to the other antibodies that were tested, SB3 binds to both intact GBS and desialylated GBS suggesting that it may recognize a completely different component of the capsid (Pincus et al., 2012). Taken together, it appears that the capsid of type III GBS is organized in immunogenic saccharide clusters that are stabilized by the sialic acid Neu5Ac.

In like manner a variety of different recombinant soluble human Siglecs bind differentially to various GBS serotypes, despite the fact that they all contain the same Neu5Acα2-3Galβ1-4GlcNAc sequence (Carlin et al., 2007, 2009). These are further examples wherein a single glycan sequence is necessary but not sufficient for recognition by various antibodies and GBPs. The best explanation for these findings is that unique CSPs are being recognized.

8.2. Antibody recognition of *N*-glycans clusters on HIV gp120

While the focus of this review has been sialylated CSPs, a recent prominent example involving mannose-containing glycans deserves special attention. Highly ordered mannose clusters on HIV gp120 monomers are comprised

of an intrinsic patch of unprocessed $Man_{5-9}GlcNAc_2$ glycans, flanked by a rim of complex glycans, surrounded by trimer-associated Manα1-2Man patch. This clustered mannose patch is recognized by the 2G12 neutralizing antibody. Additional CSPs on gp120, comprised of several N-linked oligomannose clustered with a short peptide sequence are recognized by other broadly neutralizing antibodies, PGT 125–128, PGT 130, and PG9. The HIV envelope protein Env is comprised of two noncovalently bound glycoproteins: the transmembrane subunit gp41 and the external subunit gp120. The gp120 subunit has an average of 25 N-linked glycosylation sites, and the position of these sites is highly conserved between HIV isolates and clades (Li et al., 2009; Scanlan et al., 2007a; Zhang et al., 2004). The glycosylation of gp120 results in a tightly packed cluster of oligomannose, which is evidently not found on mammalian cell surfaces (Balzarini, 2006). The formation of this unique cluster starts with the addition of $Glc_3Man_9GlcNAc_2$ precursor to the N-linked glycosylation sites in the ER. Glucose residues are then cleaved by α-glucosidases I and II to produce $Man_9GlcNAc_2$ or D2-, D3-$Man_8GlcNAc_2$, depending on the cell type (Bonomelli et al., 2011; Doores et al., 2010; Sanders et al., 2002). Further processing of the oligomannose chains to produce complex glycans is limited by the high density of mannose residues and by steric consequences of gp120 trimerization. As a result only a limited area on the gp120 trimer is accessible for glycan processing. Indeed, mass spectrometry analysis confirms that less than 2% of the total glycans on gp120 are complex glycans (Scanlan et al., 2007a; Zhu et al., 2000). Taken together, it was suggested that each gp120 monomer has an intrinsic patch of unprocessed $Man_{5-9}GlcNAc_2$ glycans, flanked by a rim of complex glycans, surrounded by trimer-associated Manα1-2Man patch (Li et al., 2009; Scanlan et al., 2007a; Zhu et al., 2000). This uniquely conserved HIV-specific glycosylation pattern was found in HIV isolates from clades A, B, and C and in simian immunodeficiency virus (SIV) (Scanlan et al., 2007a).

Given that the clustered oligomannose patch appears to be conserved for HIV viruses and does not exist on mammalian cells, it is potentially a good target for vaccine design. It was shown that the broadly neutralizing 2G12 human anti-HIV antibodies bind to the intrinsic mannose patch at the center of this complex mannose cluster (Calarese et al., 2003; Li et al., 2009; Scanlan et al., 2002). The high mannose patch can be mimicked on cultured cells by inhibition of $Man_9GlcNAc_2$ trimming with kifunensine, a mannose analog that competitively inhibits type 1 ER and Golgi α-mannosidases. The 2G12 antibody bound to the abundant oligomannose-type glycans on

kifunensine-inhibited human 293T cells, and to human CEACAM1 protein that was expressed in these cells. Like gp120, this protein has a 120 kDa highly mannosylated extracellular domain, when it was expressed in 293T cells in the presence of kifunensine (Scanlan et al., 2007b). Although 2G12 recognizes terminal Manα1-2Man residues on high mannose glycans (Sanders et al., 2002; Scanlan et al., 2002, 2007b), the spatial organization of mannose in the cluster is important for the binding and neutralization of HIV isolates with 2G12 (Astronomo et al., 2010; Scanlan et al., 2007b). Antibodies that were generated against high mannose clusters were ineffective against primary HIV isolates. For example, *Saccharomyces cerevisiae* yeast with a deletion in the α1-3 mannosyltransferase gene ΔMnn1, or a deletion of three genes (ΔOch1 ΔMnn1 ΔMnn4) in the N-linked glycosylation pathway, expresses high density of $Man_9GlcNAc_2$ or $Man_8GlcNAc_2$ oligosaccharides, respectively. Although 2G12 antibody can bind these yeasts, immunization with these mutants results in antibodies that bind gp120 but fail to neutralize primary HIV isolates (Agrawal-Gamse et al., 2011; Dunlop et al., 2010; Luallen et al., 2008). Similarly, a mannose binding molecule, cyanovirin-N, binds to gp120 and can neutralize laboratory-adapted strains, but only very weakly neutralize primary HIV isolates (Scanlan et al., 2007a). Many attempts were made to mimic the mannose cluster on gp120 protein by chemically synthesizing compounds containing multiple Man_{9}-$GlcNAc_2$ chains attached to a rigid scaffold at various densities (Astronomo et al., 2010; Joyce et al., 2008; Krauss et al., 2007). All these compounds were recognized by 2G12 antibody, and induced robust carbohydrate-specific antibody responses in immunized animals, but failed to elicit broadly neutralizing antibodies against HIV.

In addition to 2G12, other broadly neutralizing antibodies for HIV were shown to interact with the densely packed oligomannose Man_8 and Man_9 on the gp120 protein. The PGT monoclonal antibodies 125–128 and 130 bound specifically to $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ on glycan array (Pejchal et al., 2011; Walker et al., 2011). Unlike 2G12, binding of these antibodies to pg120 can be competed out with Man_9 but not with monomeric mannose or Man_4, suggesting that they bind to a different epitope (Walker et al., 2011). Despite forming 11–16 hydrogen bonds with a number of terminal mannose on gp120, only two of the $Man_{8-9}GlcNAc_2$ oligosaccharides (position 332 and/or 301) are critical for binding of PGT 127–128 antibodies (Pejchal et al., 2011; Walker et al., 2011). In addition, a short peptide at the C-terminal V3 stem is required for binding (Pejchal et al., 2011). This suggests that PGT 127–128 antibodies bind to

a specific CSP comprised of several mannose residues and a peptide, which is stabilized by one or two N-linked oligomannose. Another broadly neutralizing antibody, PG9, binds to a cluster comprised of $Man_5GlcNAc_2$, $Man_4GlcNAc_2$, and a β-sheet strand (McLellan et al., 2011).

Taken together these findings suggest that the spatial organization of mannose residues in the intrinsic patch is stabilized by the flanking glycans. Thus, in order to generate high potency HIV-broadly neutralizing antibodies the entire mannose CSP must be taken into account.

9. GLYCOSYNAPSES: CELL INTERACTIONS AND SIGNALING VIA CSPs

Glycosynapses are a type of cell surface microdomain that are stabilized by carbohydrate–carbohydrate interactions, and mediate adhesion and signaling between two cells or between cells and the ECM (Hakomori, 2002; Hakomori and Handa, 2002; Todeschini and Hakomori, 2008). Glycosynapse microdomains are classified by their content and function to three types: Type I are comprised of GSLs clustered with signaling proteins; Type II are comprised of O-linked mucin-type glycoproteins clustered with Src family kinases; and Type III are comprised of GSLs, N-glycosylated adhesion receptor, tetraspanins, and signaling proteins (Hakomori, 2004; Todeschini and Hakomori, 2008). All of the glycosynapses are stabilized by *cis* carbohydrate–carbohydrate interactions between GSLs, and are distinctively different from lipid rafts and caveolae microdomains (Hakomori, 2003).

In Type I glycosynapses, the glycosylated N-terminal domain of a GSL cluster stabilizes a saccharide cluster on the plasma membrane and GSL lipid tails interact with signaling proteins. These glycosynapses mediate cell–cell adhesion and signaling either through carbohydrate–protein interactions or carbohydrate–carbohydrate interactions between two cells (Todeschini and Hakomori, 2008). The carbohydrate–protein-mediated adhesions typically involve a "lock and key" type interaction between gangliosides and GBPs, for example, galectin-1 interaction with GM1 microdomain (Hakomori, 2004; Siebert et al., 2005). In contrast, carbohydrate–carbohydrate-mediated cell–cell adhesion is described as a zipper or a gear wheel in which a perfect fit between interacting carbohydrates is required for adhesion (Spillmann and Burger, 1996). Carbohydrate–carbohydrate interactions are stabilized primarily by van der Waal's forces, although dipole–dipole and hydrogen bonds may also form between the molecules. In most cases,

carbohydrate–carbohydrate interactions require the presence of bivalent cations (Ca^{2+} or Mg^{2+}) (Spillmann and Burger, 1996; Todeschini and Hakomori, 2008). Examples of carbohydrate–carbohydrate-mediated adhesion by type I glycosynapses include: Le^x-to-Le^x (Galβ1-4(Fucα1-3) GlcNAc) interactions between two cells to induce auto-aggregation of mouse tetratocarcinoma F9 cells (Eggens et al., 1989; Hakomori, 2004); GM3 (Neu5Acα2-3Galβ1-4Glcβ1-Cer) on B16 mouse melanoma cells interaction with Gg3 (GalNAcβ1-4Galβ1-4Glcβ1-Cer) on L5178 mouse lymphoma cells (Kojima and Hakomori, 1989), or with lactosylceramide (Galβ1-4Glcβ1-Cer) on endothelial cells (Kojima and Hakomori, 1991); Le^y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) in mouse uterus epithelium with blood group H antigen (Fucα1-2Gal) (Zhu et al., 1995); and human embryonal carcinoma cells aggregation by interaction of globoside Gb4 (GalNAβ3Galα4Galβ4Glcβ1Cer) *cis* with Gb4, GalGb4 or *trans* with nLc_4 (Le^x precursor, Galβ1-4GlcNAc) on a neighboring cell (Song et al., 1998).

Type II glycosynapses are comprised of O-linked mucin-type glycoproteins clustered with Src family kinases, and are not very well studied. The best example is the MUC1-containing microdomains that were isolated from low-density membrane fraction of T cells (Hakomori, 2004). These microdomains include three mucin-type glycoproteins (MUC1, PSGL-1, and CD45), and three Src family kinases (Yes, Fyn, and lck56) (Agrawal et al., 1998a,b). MUC1 proteins cluster at the leading edge of activated T cells, an area that is also associated with gangliosides (Correa et al., 2003). MUC1 interactions with receptor proteins may induce signaling to mediate T-cell activation and migration (Hakomori, 2004). PSGL-1 mediates selectin binding (see Section 10.2) and CD45 is involved in regulating T-cell activation via the TCR (Altin and Sloan, 1997). Taken together the data indicate that type II glycosynapses are involved in carbohydrate-mediated cell adhesion and signaling (Hakomori, 2004).

Type III glycosynapses mediate cell–ECM adhesion, and are comprised of GSLs, an N-glycosylated adhesion receptor (e.g., integrin), tetraspanin, and signaling proteins (Todeschini and Hakomori, 2008). Integrins are a family of α/β hetrodimeric proteins that mediate cell–cell and cell–ECM adhesion (Scales and Parsons, 2011). Integrins that bind to ECM components (laminin, collagen, and fibrinogen) often associate with tetraspanins, which are palmitoylated and N-glycosylated transmembrane proteins (Bassani and Cingolani, 2012). *Cis* interactions among integrins, tetraspanin, and gangliosides depend on the N-glycosylation level on all the glycosynapse

components (Ono et al., 2000, 2001; Todeschini and Hakomori, 2008). Transmembrane signaling proteins are also stabilized by interactions between the N-linked glycans, cytoplasmic signaling proteins are stabilized by interactions with the GSLs lipid tail. Examples of type III glycosynapses include GM3/tetraspanin CD9 inhibition of cell motility and proliferation through modulation of integrin and FGF receptor function (Hakomori, 2004; Todeschini and Hakomori, 2008); and GM2/GM3/tetraspanin CD82 complex inhibit activation of hepatocyte growth factor by the tyrosine kinase receptor Met, and cross-talk with integrin α3β1 (Todeschini and Hakomori, 2008).

In conclusion, glycosynapse microdomains are comprised of GSLs, glycoproteins, and signaling proteins that are stabilized by *cis* carbohydrate–carbohydrate interactions. Such CSP-containing glycosynapses are involved in carbohydrate-dependent cell–cell and cell–ECM adhesion, and induce cell activation, motility, and growth.

10. RECOGNITION OF COMBINED SACCHARIDE–PEPTIDE PATCHES

In addition to specific glycan epitopes, several amino acids of the core protein can be required for receptor recognition, in some cases, forming an oligosaccharide–peptide patch. Sometimes the amino acids are sulfated and may be substituted for sulfated oligosaccharides. Here, we present two examples of oligosaccharide–peptide patch recognition.

10.1. *O*-sialoglycopeptide recognition by paired immunoglobulin-like receptors

The ligand for paired immunoglobulin-like receptors type 2 (PILRs) is comprised of a cluster of sialylated core 1-type O-linked glycan chain and a protein determinant (Sun et al., 2012; Wang et al., 2008a). PILRs belong to one of the paired receptor family, which consists of a pair of inhibiting (PILRα) and activating (PILRβ) receptors, are predominantly expressed in immune cells (Mousseau et al., 2000). One of the best studied ligands for PILRs is mouse CD99 (mCD99), an O-glycosylated protein that is expressed on all leukocyte lineages, and highly expressed on activated T cells (Schenkel et al., 2002; Shiratori et al., 2004; Tabata et al., 2008). O-glycosylation of threonine residues 45 and 50 on mCD99 is required for recognition by both PILRα and PILRβ. However, PILRα binding to mCD99 is not affected by a single alanine mutation of either threonine

45 (T45A) or threonine 50 (T50A). In contrast, mutation of T45A inhibits the binding of PILRβ to mCD99 (Tabata et al., 2008; Wang et al., 2008a). It was further shown that the O-glycosylation is likely comprised of a sialylated core 1-type structure. Both core 2 branching and desialylation inhibit PILRs binding to mCD99 (Sun et al., 2012; Wang et al., 2008a). Although sialylated core 1 structures are likely found on additional surface proteins on the cells, PILRs do not bind to parental cells that were not transfected with mCD99. Furthermore, PILRs do not bind sialylated oligomeric glycans on ELISA-like binding assay (Sun et al., 2012). Thus sialylated core 1 structure by itself is not the ligand for PILR binding (Wang et al., 2008a).

In addition to the sialylated core 1 structure, the PILR ligand is comprised of an adjacent protein determinant. Both mouse and human PILRα bind mCD99 but not human CD99 (hCD99), although both present similar glycosylation structures ($Neu5Ac_{1\text{-}2}Hex_1HexNAc_1$) (Sun et al., 2012). As mentioned above, mCD99 has two adjacent O-glycosylation sites (Thr-45 and Thr-50) each independently sufficient to promote PILRα binding (Tabata et al., 2008; Wang et al., 2008a). In contrast, hCD99 has only one occupied O-glycosylation site on Thr-41, which corresponds to Thr-45 site on mCD99. Insertion of the mCD99 amino acids region between Pro-46 and Thr-50 (^{46}PKAPT^{50}) to hCD99 was sufficient for PILRα binding (Sun et al., 2012). Since only one O-glycosylation site is sufficient to promote PILRα binding, the PILRα ligand is comprised of one sialylated core 1-type O-glycosylation and an adjacent protein motif (Sun et al., 2012). Additional PILRs ligands that have similar binding motifs are PILR-associating neural protein (Kogure et al., 2011), neuronal differentiation and proliferation factor-1, collectin-12 (Sun et al., 2012), and herpes simplex virus-1 glycoprotein B (Wang et al., 2009).

10.2. P- and L-selectin recognition of clustered *O*-sialoglycan-sulfated epitopes

P- and L-selectin bind to a clustered patch comprised of sialic acid, fucose, and sulfate residues at a specific spatial orientation on their protein ligands. Selectins are transmembrane glycoproteins that mediate cell–cell interactions, P-selectin is expressed on activated vascular epithelium and platelets, L-selectin is constitutively expressed on leukocytes, and E-selectin is expressed on sites of inflammation (Konstantopoulos and Thomas, 2009; Varki, 1994, 1997). Selectins weakly interact with sialylated, fucosylated lactosaminoglycans such as Sialyl-Lewis^x (Siaα2-3Galβ1-4(Fucα1-3) GlcNAcβ-, SLe^x), or its isomer Sialyl-Lewis^a (Siaα2-3Galβ1-3(Fucα1-4)

GlcNAcβ-, SLe^a). In addition, P- and L-selectins can also bind certain sulfated molecules like heparin (Norgard et al., 1993b; Varki, 1994). In contrast, E-selectin binding is sulfation-independent, and requires sialylated fucosylated N-linked glycans (Kanamori et al., 2002; Lenter et al., 1994; Levinovitz et al., 1993; Zöllner and Vestweber, 1996). Careful analysis of selectin binding requirements to their natural protein ligands reveals that the composition, charge, and spatial organization of monosaccharides and sulfate epitopes are important for binding. The cluster may be formed by oligosaccharide and sulfated peptide (e.g., P-selectin glycoprotein ligand-1 (PSGL-1)), more than one oligosaccharide chain (e.g., CD24) or form on a single oligosaccharide chain (e.g., 6-sulfo-SLe^x on GlyCAM-1). Here, we discuss the former two clusters.

PSGL-1 is a dimeric mucin type glycoprotein that was originally identified as P-selectin ligand (Moore et al., 1992) but can interact with L- and E-selectins as well (McEver and Cummings, 1997; Moore, 1998). The extracellular domain of human PSGL-1 is densely O-glycosylated with *O*-glycans that are neutral, mono- or disialylated forms of the core 2 tetrasaccharide Galβ1-4GlcNAcβ1-6(Galβ1-3)GalNAcα1-OH. Core 2 modifications include SLe^x and α1,3Fuc structures (Moore, 1998; Wilkins et al., 1996). In addition to the extensive glycosylation, PSGL-1 contains up to three sulfated tyrosine residues (Tyr-46, Tyr-48, and Tyr-51) (Pouyani and Seed, 1995; Wilkins et al., 1995). Both O-linked glycosylation and sulfation are required for P- and L-selectin binding (McEver, 2002; Norgard et al., 1993b). The exact epitope for P- and L-selectin binding on PSGL-1 was identified by analysis of glycosulfopeptides corresponding to the N-terminal amino acid residues 45–61 of human PSGL-1. The synthesized glycosulfopeptides contained between 1 and 3 sulfated tyrosines, and were O-glycosylated at Thr-57 with different core 2 structures with SLe^x determinant (Leppanen et al., 2003; Leppänen et al., 2002). Both P- and L-selectin required core 2 SLe^x determinant, and a minimum of two adjacent sulfated Tyr in close proximity to the glycosylation site for binding. L-selectin binding requires the sulfated Tyr to be immediately adjacent to the glycosylation site (Tyr-48 and Tyr-51) in contrast to P-selectin that is not as selective (Leppanen et al., 2003). This distinction is interesting because L-selectin can bind to O-linked core 2 6-sulfo SLe^x structures (e.g., on GlyCAM-1 and CD34), in contrast to P-selectin which requires the sulfated Tyr for binding (Hemmerich and Rosen, 2000; Hernandez Mir et al., 2009; Kanamori et al., 2002). However, optimal binding of both P- and L-selectin was obtained only when all three

Tyr residues were sulfated. Elongation or desialylation of the glycosylated chain inhibits P- and L-selectin binding determinant (Leppanen et al., 2003; Leppänen et al., 2002), indicating that the spatial distance between SLe^x and sulfated tyrosines is important for an optimal fit in the binding pocket. This suggests the core 2 O-linked SLe^x and 2–3 sulfated tyrosines form a cluster on PSGL-1 that is recognized by P- and L-selectins.

Another example is human CD24 (heat-stable antigen), a small mucin-type GPI-anchored protein that has O-sialoglycosyl modifications on nearly half of its amino acids (Pirruccello and LeBien, 1986). Unlike PSGL-1, CD24 does not contain a sulfated Tyr residue (Aigner et al., 1997). However, certain CD24 glycoforms contain a sulfated glucuronylneolactose carbohydrate modification (HNK-1) sulfate-3GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc (Aigner et al., 1997; Chou et al., 1986; Sammar et al., 1994). P- and L-selectin bind to sulfated glycosphingolipids containing the HNK-1 epitope *in vitro* (Needham and Schnaar, 1993). O-linked glycosylation, sialylation and the HNK-1 modification are all required for P-selectin binding to CD24 (Aigner et al., 1997). In contrast, treatment with endoglycosidase F to remove N-linked glycans does not affect P-selectin binding to human CD24 (Aigner et al., 1997). Only certain glycoforms of CD24 contain SLe^x modifications (Aigner et al., 1997, 1998), but these modifications are required to mediate CD24-dependent rolling and adhesion by selectins (Aigner et al., 1998). Taken together, the data indicate that P- and L-selectin bind to a cluster of SLe^x and sulfated glucuronylneolactose on CD24.

11. EXPERIMENTAL RECREATION OF CSPs

Given how little is really understood about CSPs, recreating the composition and spatial organization of glycans in a natural CSP in an *in vitro* setting is very difficult. However useful information can be obtained by spotting glycan mixtures, neoglycoproteins, and glycans on dendrimeric scaffold onto glycan microarrays, a relatively new approach to the study of glycans (Feizi et al., 2003). In the context of CSPs, a drawback of the microarray approach is the static state of the printed glycans. The cell membrane is a fluidic lipid bilayer environment, in which lateral movement can be a significant factor in mediating multivalent interactions. In order to mimic the membrane environment more closely, fluidic microarrays have been designed. Here, we briefly present evidence for CSPs recognition in glycan microarrays and fluidic microarrays.

11.1. GBP binding to mixed glycan clusters on glycan microarrays

Microarrays are a great tool for high-throughput analysis of GBP interactions with various glycan epitopes. A large number of glycan libraries comprised of synthetic glycans, glycans isolated from natural sources or a mixture of both are currently available to researchers (Lonardi et al., 2010; Padler-Karavani et al., 2012; Rillahan and Paulson, 2011). Glycans are immobilized on these arrays by various covalent and noncovalent methods. Covalent immobilization is most commonly achieved by functionalization of the glycans with thiol or amine groups, for example, and applying to an appropriate reactive surface. This approach yields a high-density glycan surface with known orientation (Rillahan and Paulson, 2011). Noncovalent methods include glycan adsorption to nitrocellulose, oxidized polystyrene, electrostatic or hydrophobic surfaces, which yields glycans in random orientation. In addition the glycans can be biotinylated and immobilized on streptavidin-conjugated surface, or tagged with DNA and immobilized to complementary DNA (Rillahan and Paulson, 2011). Binding of GBPs to glycan arrays is influenced by the length and flexibility of the linker, the underlying glycan structures and the glycan densities. This was demonstrated by cross-comparison of lectin binding to otherwise identical terminal sialylated glycan structures between two glycan array platforms (Padler-Karavani et al., 2012). Furthermore, due to the multivalent nature of GBPs interactions with glycan epitope, the spacing (density) and orientation of glycans on the array are important factors for binding (Taylor and Drickamer, 2009; Zhang et al., 2010a).

One approach for controlling the spacing between glycan epitopes is to conjugate glycan epitopes to a carrier protein, for example, albumin, generating a multivalent glycoconjugate termed neoglycoprotein (Roy, 1996; Stowell and Lee, 1980). The neoglycoproteins are then mixed with non-glycosylated spacer proteins (e.g., BSA) at a known ratio, and the mixture is immobilized on an array (Zhang et al., 2010b). The distance between neighboring neoglycoproteins is determined by the ratio between spacer proteins and neoglycoproteins, and can be estimated from the spacer protein dimensions. For example, in a tightly packed monolayer of 1:7 neoglycoprotein to BSA mixture, on average, there should be one neoglycoprotein in the middle of each group of eight molecules. BSA dimensions are approximately $35\ \text{Å} \times 35\ \text{Å} \times 70\ \text{Å}$, with the assumption that BSA molecules adhere to the surface with the 70 Å side parallel to

the surface, the distance between two neighboring neoglycoproteins should be about 140 Å (Zhang et al., 2010a). This approach provides a distinction between two modes of multivalent binding of GBP to glycan cluster. The nonglycosylated spacer proteins will not affect multivalent binding of GBP to several glycan epitopes that are presented on a single neoglycoprotein. Conversely, the spacer protein will inhibit multivalent binding of GBP to several glycan epitopes that are presented on two (or more) different neoglycoproteins, forming a homogenous CSP (Zhang et al., 2010a). Interestingly in this array the average spacing between the centers of two neighboring neoglycoproteins (140 Å) is too large for efficient energy transfer between donor and acceptor. Critical distance for Förster resonance energy transfer (FRET) is ~90 Å (Adams et al., 1991; Ishikawa-Ankerhold et al., 2012). Thus a CSP formation does not necessarily require the core proteins to form a stable complex or to physically interact.

Binding of the plant lectin concanavalin A (Con A) to mannose and glucose was examined using this approach. ConA bound at a similar apparent K_d value to a uniform layer of Glc-α-BSA, Man-α-BSA, or Man6-BSA. ConA binding to Man6-BSA was not affected by adding BSA spacer at 1:7 Man6-BSA/BSA ratio, suggesting that ConA binds to several mannose epitopes on a single neoglycoprotein. In contrast, adding the BSA spacer at 1:7 ratio abolished ConA binding to Glc-α-BSA and to Man-α-BSA. Thus ConA forms multivalent interactions with glycans on two or more neoglycoprotein (Zhang et al., 2010a), indicating specific recognition of homogenous clustered patch of mannose or glucose. Another example for homogenous CSP binding is the binding of *Vicia villosa* lectin B4 (VVL-B4) to GalNAc. VVL-B4 binding to neoglycoproteins presenting GalNAc with various underlying structures is inhibited by adding the BSA spacer proteins (Zhang et al., 2010a). This method can be used to tease out binding of commercially available antibodies and patient serum to CSPs. For example, the binding properties of five IgM and IgG1 type monoclonal antibodies for blood group A (GalNAcα1-3(Fucα1-2)Galβ-) and A1 (GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc) were tested. All five antibodies tightly bound to blood group A and A1 epitopes on the array with low apparent K_d value (Zhang et al., 2010b). However, adding BSA spacer at 1:7 ratio abolished the binding of one of the tested antibodies (IgM) to blood group A1 epitope but not to the blood group A epitope. Two of the antibodies (IgM and IgG1) exhibit additional strong binding to

GalNAcα1-3Galβ-epitope on the array, which was abolished by adding BSA spacer (Zhang et al., 2010b).

Composition and spatial organization of naturally occurring CSPs on cells could be very complex and difficult to mimic on the above kind of microarray. Instead, spotting mixtures of two different glycans on an array provides a simplified version of heterogeneous saccharide patches. Theoretically, a mixture of 1:1 should generate a surface in which the two glycans are evenly distributed (Liang et al., 2011). Antibody or GBP binding to a mixed surface can be then compared with the binding to a uniform layer of its specific ligand. A change in the binding K_d is an indication to the effect of neighboring glycans on antibody–ligand interactions. The binding can be reduced due to steric hindrance by the neighboring glycans, can remain unaffected, or can be enhanced by the formation of a novel epitope comprised of two or more neighboring glycans (Liang et al., 2011). For example, the binding of an antibody against the glycosphingolipid SSEA3 (Stage-Specific Embryonic Antigen 3, Gb5) was tested on an array with 1:1 mixtures of SSEA3 with six other glycosphingolipids of the globo-series glycolipids (SSEA4, Globo H, Gb4, Gb3, Gb2, and Bb2) (Kannagi et al., 1983; Liang et al., 2011). With exception of the SSEA3/SSEA4 mixture, antibody binding to SSEA3 was reduced in the mixed surface presumably due to steric hindrance by neighboring glycans. Not surprisingly, the steric hindrance was directly proportional to the length of the neighboring glycan. Interestingly, however, higher antibody binding to SSEA3/SSEA4 mixture was observed compared with binding to SSEA3 alone (Liang et al., 2011). Although the antibody did cross-react with SSEA4 directly, the binding intensity to SSEA3/SSEA4 mixture was higher than the intensity of the average binding to SSEA3 and to SSEA4. This suggests that a cluster of SSEA3 and SSEA4 generates the preferred ligand for the antibody.

In another approach, two types of oligomannose (Man4 and Man9) were conjugated to a second-generation dendrimetric scaffold at different ratios. The oligomannose dendrimers were spotted on a microarray, creating clusters of oligomannose with 9:0, 0:9, 6:3, 3:6, and 5:4 Man4:Man9 ratio (Liang et al., 2011; Wang et al., 2008b). The HIV neutralizing antibody, 2G12 preferably bound to the 5:4 dendrimer with half of the binding K_d compared with the other dendrimers (13.47 nM compared with 30.34–47.4 nM). In theory, at 5:4 ratio every Man9 is flanked with Man4 thus potentially presenting a new clustered epitope comprised of both oligomers (Liang et al., 2011).

11.2. Combinatorial glycoarrays and fluidic glycan microarrays

Combinatorial glycoarrays and lipid microarrays are another approach to study binding to glycosphingolipid mixtures. In examples of this approach, a mixture of glycolipids/lipids is printed onto a polyvinylidene difluoride membranes affixed to glass slides. The lipid tail binds noncovalently to the hydrophobic surface and the oligosaccharide head group is free to interact with GBPs and antibodies (Kanter et al., 2006; Rinaldi et al., 2012). The combinatorial glycoarray is used to compare the interactions with a single ganglioside compared to interactions with its heterodimeric ganglioside mixtures (Rinaldi et al., 2012). Lipid microarrays typically include lipid extracts from a variety of tissues, biological fluids, and cells (Kanter et al., 2006).

Combinatorial glycoarrays were used to identify antibodies against ganglioside complexes in sera from neuropathy patients (see Section 5.1). In addition, combinatorial glycoarrays were used to study the binding of certain bacterial toxins and lectins to gangliosides and ganglioside complexes. For example, the gangliosides GD1b and GT1b are known to be part of the binding complex of Tetanus neurotoxin fragment (TeNT HC) on neuronal cells, and were shown to colocalize in immunofluorescence studies (Deinhardt et al., 2006). In agreement with these findings, TeNT HC binds very strongly both to GD1b and to GT1b on the combinatorial glycoarray and the binding is not affected by ganglioside complex formation. In contrast, TeNT HC strongly binds to GQ1b on the glycoarray, but does not bind to GQ1b/GM2 mixture (Rinaldi et al., 2009). Interestingly, although TeNT HC does not bind to the single gangliosides GD3, GM1, GD1a, or GT1a, it reacts strongly with heterodimers of these gangliosides with GD3, suggesting the formation of a neo binding epitope for TeNT HC in the GD3-containing ganglioside complexes (Rinaldi et al., 2009, 2010). Other evidence to the formation of GD3-containing complexes comes from the binding pattern of human Siglec-7-Fc to the combinatorial glycoarrays. Human Siglec-7 is a CD33-related Siglec that preferably binds to Neu5Acα2-8Neu5Ac containing structures, such as GD3 (Crocker et al., 2007). Siglec-7-Fc strongly binds to GD3 on the glycoarray, but does not bind to the GD3 when it is mixed with GM1, GD1a, or GT1a, suggesting that the GD3 epitope is masked in the cluster probably by the formation of a neo epitope (Rinaldi et al., 2009, 2010).

As discussed above, in most glycan microarrays the glycan epitopes are immobilized to the surface of the array either covalently or noncovalently. However, the cell membrane is a fluidic lipid bilayer environment, in which lateral movement is a significant factor in mediating multivalent interactions

(Yamazaki et al., 2005; Zhu et al., 2009). Several approaches have been taken to generate lipid microarrays (Brian and McConnell, 1984; Castellana and Cremer, 2007; Groves and Boxer, 2002; Tanaka and Sackmann, 2005). Lipid bilayer platform technology is the most robust and quantitative method (Deng et al., 2008). In this approach cholesterol is dispersed and immobilized by covalent linking to a brush layer of poly(ethyleneglycol) (PEG). Small unilamellar vesicles (SUV) are then deposited onto the PEG-cholesteryl surface to form a lipid bilayer. The cholesterol molecules stabilize the lower leaflet of the bilayer while maintaining the fluidity of the outer layer (Deng et al., 2008). Different glycoproteins and glycolipids can be incorporated into the SUV at varying concentrations in order to form a fluidic glycan microarray (Bricarello et al., 2010; Deng et al., 2008; Zhu et al., 2009).

Fluidic glycan microarrays were used to study the interaction of pathogens with their known glycan receptors. For example, type 1 adhesin FimH, located at the tip of *Escherichia coli* pili, bind to mannose on host cells (Harris et al., 2001; Krogfelt et al., 1990; Schembri and Klemm, 1998). *E. coli* binding to a density gradient fluidic array of mannose-linked lipids at densities ranging from 0 to 0.3 nm^{-2} was quantified (Zhu et al., 2009). At a critical mannose concentration of 0.1 nm^{-2}, the number of bound bacteria was increased by an order of magnitude. At this concentration the inter-mannose distance is ~4 nm, which is 2 orders of magnitude shorter than the distance between two pili. Thus the binding of FimH to the array switched from monovalent binding to a trivalent binding (Zhu et al., 2009), suggesting that clustered mannose is a preferred ligand for FimH.

12. CONCLUSIONS AND PERSPECTIVES

The idea that glycans can cluster into discreet patches, and that these CSPs can influence glycan recognition and function was proposed two decades ago as a possible explanation for the extraordinary specificity of selectins for certain ligands, despite their recognition of commonly occurring glycans (Varki, 1994). In this review we present an abundance of indirect evidence to support the existence of CSPs on proteins, cells and pathogens, and evidence that glycan clustering influences their recognition by GBPs. It is likely that glycan microdomains are mostly stabilized by carbohydrate–carbohydrate interactions, which are too weak to withstand the standard biochemical methods used to study protein–protein interactions (e.g., immunoprecipitation). Furthermore, the spatial organization of

glycans in CSPs is critical for GBP recognition. Attempts to isolate them for study *in vitro* inevitably disperse the clusters (e.g., CSPs on GBS III capsid, Section 8.1). Imaging of the relative positions of glycans in CSPs is also very difficult, since imaging typically requires binding of antibodies and GBPs to a target epitope. The density and distribution of glycans in CSPs may mask recognition of individual glycans, and thus prevent their visualization within the CSP (e.g., ganglioside complexes, Section 5). Alternate approaches that directly label the glycans (using click chemistry Baskin et al., 2007 or periodate oxidation and aniline-catalyzed oxime ligation Ramya et al., 2013) also alters the glycans and potentially changes the organization of the CSP and its interaction with GBPs. Recreating natural CSPs *in vitro* and on glycan or lipid arrays is a major challenge, though technology is improving (see Section 11). Screens of antibody and GBP binding to arrays containing glycan mixtures will eventually provide a reference database of naturally occurring glycan epitopes and the molecules that bind them in their native conformation. Progress toward this goal will require advances in glycan microarray technology and perhaps even new ideas about how to generate an array of CSPs with realistic clustering properties.

Obtaining direct physical evidence of CSP formation will require technologies that do not rely on protein/antibody detection, or chemical modification of the glycans. Such methods may include crystallography, nuclear magnetic resonance (NMR) spectroscopy, or advanced microscopy methods. X-ray crystallography is not well suited to study structure of heavily glycosylated proteins such as mucins, because the heterogeneity and flexibility of glycan bonds prevents crystallization (Gerken, 1993). However, 2D crystallography methods can now visualize two-dimensional crystals of membrane proteins in a lipid environment (Kang et al., 2013; Raunser and Walz, 2009) and on living cells (Gualtieri et al., 2011). Glycans in CSPs are stabilized by *cis* interactions, so their movement should be constrained relative to glycans that are not associated with CSPs. Thus it may be possible to adapt 2D crystallography for the structural analysis of CSPs on membranes. NMR spectroscopy is another experimental tool to study glycan structure at atomic resolution. This method is typically used to analyze purified glycans or a simple mixture of glycans in solution using ^{1}H and ^{13}C isotopes (Shriver et al., 2012). Although the structure of the glycosaminoglycan heparan sulfate has been studied using NMR, the heterogeneity of glycans on mucin-like proteins may be too overwhelming for this method. Thus study of CSPs on proteins using liquid state NMR is very challenging. Recent developments in solid-state NMR enable analysis of proteins in the

cell membrane. To compensate for the high signal-to-noise ratio and increase sensitivity, proteins are labeled with fluorine (^{19}F), which is scarce in natural membranes (Koch et al., 2012). However, while the resolution is not yet sufficient to study CSPs, glycan labeling with heavy isotopes in combination with a high power energy source (e.g., synchrotron) will perhaps allow the study of CSPs on membranes.

Direct visualization of CSPs on the plasma membrane may become possible with improved high-resolution microscopy methods for imaging unlabeled hydrated samples. Advances in atomic force microscopy (AFM) and near field scanning optical microscopy allow imaging of single molecules on the plasma membrane with nanometer spatial resolution (Betzig and Trautman, 1992; Hinterdorfer et al., 2012; van Zanten et al., 2010). Using these methods enabled imaging of the spatial organization of peptidoglycan in living bacteria, nanodomains in yeast membranes, and the spatial organization of molecules in lipid rafts (reviewed in Hinterdorfer et al., 2012). In addition, the thickness and spatial distribution of glycocalyx in the plasma membrane of cultured cells was visualized using a combination of AFM and light microscopy (Bai and Wang, 2012). However, the resolution is currently not sufficient for CSP imaging and further developments are needed to enable analysis of glycan distribution within the CSP.

Considering the huge diversity of glycans, their biochemical properties, and their important role in cellular recognition and binding it is very likely that glycans are often organized into meaningful clusters of the kind discussed here. While current technology does not allow for direct imaging of CSPs, their existence and biological importance can be inferred from the wealth of evidence for CSP-specific recognition presented in this review.

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