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Sugar-Binding Proteins from Fish: Selection of High Affinity “Lambodies” That Recognize Biomedically Relevant Glycans

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Abstract

Glycan-binding proteins are important for a wide variety of basic research and clinical applications, but proteins with high affinity and selectivity for carbohydrates are difficult to obtain. Here we describe a facile and cost-effective strategy to generate monoclonal lamprey antibodies, called lambodies, that target glycan determinants. We screened a library of yeast surface-displayed (YSD) lamprey variable lymphocyte receptors (VLR) for clones that can selectively bind various biomedically important glycotopes. These glycoconjugates included tumor-associated

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Supporting Information Binding selectivity of VLRB.aGPA.23 profiled with two glycan arrays. An Excel Spreadsheet with the raw data and complete list of array components on each array: Supplementary Spreadsheet 1A (CFG array) and 1B (neoglycoprotein array). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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carbohydrate antigens (Tn and TF α), Lewis antigens (LeA and LeX), *N*-glycolylneuraminic acid, targets of broadly neutralizing HIV antibodies (poly-Man9 and the HIV gp120), and the glycoproteins asialo-ovine submaxillary mucin (aOSM) and asialo-human glycophorin A (aGPA). We isolated clones that bind each of these targets in a glycan-dependent manner and with very strong binding constants, for example, 6.2 nM for Man9 and 44.7 nM for gp120, determined by surface plasmon resonance (SPR). One particular lambdy, VLRB.aGPA.23, was shown by glycan array analysis to be selective for the blood group H type 3 trisaccharide (BG-H3, Fuc α 1-2Gal β 1-3GalNAc α), aGPA, and TF α (Gal β 1-3GalNAc α), with affinity constants of 0.2, 1, and 8 nM, respectively. In human tissue microarrays this lambdy selectively detected cancer-associated carbohydrate antigens in 14 different types of cancers. It stained 27% of non-small cell lung cancer (NSCLC) samples in a pattern that correlated with poor patient survival. Lambdies with exquisite affinity and selectivity for glycans may find myriad uses in glycobiology and biomedical research.

Glycosylation of proteins is the most common and diverse form of post-translational modification. Other important glycans are the glycolipid components of cell membranes. Glycans can profoundly affect the structure and function of glycoproteins and are critical in a wide range of normal and pathological processes, for example, in cell-cell adhesion, fertilization, inflammation, and malignant transformation.¹ Carbohydrate-binding proteins have therefore enormous utility as tools to monitor the expression of glycans for a variety of basic research and clinical applications. For instance, a number of carbohydrate-binding lectins and mammalian antibodies have been used to detect expression of tumor-associated carbohydrate antigens for diagnostic and prognostic purposes.²⁻⁴ Glycan-binding proteins can also be used for *in vivo* applications, such as targeting specific cells and tissues for imaging, for drug delivery, and to control carbohydrate-mediated processes. Unfortunately, most readily available glycan-binding proteins, such as plant lectins and mammalian antibodies, typically display either broad specificity or poor affinity for carbohydrate antigens. For example, most monoclonal antibodies (mAb) that specifically recognize the Thomsen-Friedenreich pancarcinoma tumor-associated carbohydrate antigen (TF α , Gal β 1-3GalNAc α 1-Ser/Thr) are of the IgM isotype, which are large antibodies with relatively low affinity, and therefore have limited clinical utility.⁴ Furthermore, carbohydrate-binding proteins are only available for a tiny fraction of over 7,000 glycan determinants estimated for the human glycome.⁵ Therefore, methods to generate tailored glycan-binding proteins with high affinity and selectivity for any glycan of interest could advance the field.

A number of strategies have been evaluated for obtaining glycan-binding receptors. The most commonly used approach involves immunizing animals with an appropriate glycan or glycoconjugate to raise mAbs,^{3,6} but this process can be slow and labor intensive. This approach works well in certain cases, but many interesting glycans are conserved among species and, therefore, could be nonimmunogenic. Other approaches include directed evolution of lectins^{2,7} and of single chain Fv (scFv) antibodies,^{8,9} small peptide carbohydrate receptors,¹⁰ and carbohydrate-binding aptamers,¹¹ but these have not proven to be general methods and have not gained widespread use. Therefore, a simple, efficient, and general method is still critically needed.

Here we describe a strategy for selection of glycan-binding monoclonal lamprey antibodies, which we call lambdies. A YSD library of VLRs, which are the unique lamprey and hagfish leucine-rich repeat based antibodies,¹²⁻¹⁴ was screened for clones that can selectively bind glycans and glycoproteins. In contrast to other methods, this strategy readily produced high affinity glycan-binding clones with selectivity and affinity that rivals that of most naturally occurring lectins and conventional antibodies.

RESULTS AND DISCUSSION

Isolation of Lambdies against Tumor-Associated Carbohydrate Antigens

Practically all types of human tumor cells express glycoproteins with aberrant glycosylation patterns.^{1,4} The best studied cancer-specific carbohydrates are truncated O-glycans such as the T-nouvelle antigen (Tn, GalNAc α 1-Ser/Thr) and TF α , both considered pancarcinoma antigens that uniquely decorate mucin-type glycoproteins in about 90% of human cancer cells but are distinctly absent from nearly all normal tissues. We began our study by isolation of clones that can bind TF α with tumor reactivity, using two types of antigens: a synthetic polyacrylamide (PAA) glycoconjugate of TF α (Figure 1) and aGPA, a human erythrocyte membrane glycoprotein whose native form is decorated with 16 sialyl-TF α structures.¹⁵ We alternated between TF α and aGPA as antigens used in consecutive rounds of enrichment, using TF α for the first of two rounds of magnetic-activated cell sorting (MACS) and for the first of two rounds of fluorescence-activated cell sorting (FACS), switching to aGPA in the second MACS and in the final FACS, when we sorted individual clones. Nine of the 15 clones that we isolated were of unique sequence (60%), and some of these showed high selectivity for aGPA over native GPA (Figure 2), unreactive to sialylated TF α structures that are common carbohydrate structures in normal mucin-type glycoproteins. In addition, some of the clones could discriminate between TF α and TF β , the Gal β 1-3GalNAc β anomeric structure of the GM1 ganglioside, which is expressed in a variety of normal human cells, including natural killer cells that are important immune effector cells. Other clones were less specific, also binding TF β and fucose, which is another glycan we used in the initial assessment of selectivity of these clones. Clone VLRB.aGPA.23 revealed the desired selectivity for aGPA and TF α and was chosen for in-depth analysis of its ligand binding properties.

Binding Selectivity of VLRB.aGPA.23

We then profiled optimal carbohydrate ligands and assessed the selectivity of this lambdy using two printed glycan microarrays. One array, from the Consortium for Functional Glycomics (CFG),¹⁶ consisted of 611 glycan structures. BG-H3 (Fuc α 1-2Gal β 1-3GalNAc α) and the H disaccharide (BG-H, Fuc α 1-2Gal β) were the main reactive structures (Figures 1 and 3 and Supporting Information Spreadsheet 1A). The second array consisted of 215 neoglycoproteins and glycoproteins,¹⁷ and on this array predominantly aGPA and glycopeptides containing TF α -Serine structures were recognized (Supporting Information Spreadsheet 1B). In agreement with the initial selection data, no binding to GPA and very weak binding to TF β were observed. TF α -Serine was not present on the CFG array, and BG-H3 and the BG-H disaccharide were not present on the second array.

The large collection of glycans and the complementary diversity on the two arrays provided detailed structural information on binding specificity of VLRB.aGPA.23. The preferred ligand for this lambdy appeared to be BG-H3, a fucosylated TF α structure. Although the BG-H disaccharide was also bound, other fucosylated blood group H antigens, including blood group H1, H2, H4, and H6, were not recognized on the arrays, nor were extended structures, such as blood group A or blood group B. Interestingly, not all oligosaccharides with a terminal BG-H3 determinant were recognized equally. BG-H3 trisaccharides attached to oligosaccharide chains, for example, Fuc α 1-2Gal β 1-3GalNAc α 1-3 (Fuc α 1-2)Gal β 1-4Glc β -Sp0, were not bound by the lambdy. In addition, BG-H3 attached to a threonine (Fuc α 1-2Gal β 1-3GalNAc α -Sp14) displayed reduced binding as compared to a simple linker (Fuc α 1-2Gal β 1-3GalNAc α -Sp8). This suggests that the moiety at the reducing end of the BG-H3 may also influence recognition. While the terminal fucose contributes to binding, it does not appear to be essential. In particular, the Gal β 1-3GalNAc α

disaccharide (TF α substructure) lacking the terminal fucose was also recognized, but only when attached to a serine. The disaccharide fragment alone (not attached to serine; Gal β 1-3GalNAc α -Sp8 or Sp16) was bound only very weakly by the lambdy, and this disaccharide was not recognized at all when attached to threonine (Gal β 1-3GalNAc α -Sp14). Again, this data suggests that the moiety to which the BG-H3/TF α -Serine is attached can influence recognition; however, additional studies will be required to more fully characterize the impact of variations beyond the primary epitope.

The glycan array data demonstrates that VLRB.aGPA.23 possesses a high degree of selectivity for BG-H3 and TF α -Serine glycans. Although there was some cross-reactivity with other glycans (e.g., the non-human structures Gal β 1-2Gal β -Sp8 and Gal β 1-6Man α), the overall selectivity of this lambdy was better than most naturally occurring lectins and antibodies.^{18,19} For example, mAb JAA-F11, an anti-TF α IgG, was profiled with a CFG microarray of 200 glycans and shown to react mainly with four structures.²⁰ These were TF α , the core-2 trisaccharide [Gal β 1-3(GlcNAc β 1-6)GalNAc α], 6-sialyl-TF α [Gal β 1-3-(Neu5Ac β 2-6)GalNAc α], and 6-LacNAc-Tn [Gal β 1-4GlcNAc β 1-6GalNAc α]. Lambdy VLRB.aGPA.23 reacted with a narrower spectrum of related structures, and was non-reactive to sialyl-TF α , which is a common O-glycan in normal mucin-type glycoproteins.

Binding Affinity of VLRB.aGPA.23

To further evaluate the affinity and selectivity of this lambdy, SPR was used to measure binding of TF α , aGPA, and BG-H3 (Figure 4). The dimeric lambdy was captured via an anti-His tag mAb immobilized on the surface of the Biacore sensor chip. At concentrations of glycoconjugates from 156 pM to 40 nM, the binding kinetics were found to fit very well a 1:1 Langmuir model (χ^2 0.075, T -values > 100, U -values 3), with calculated affinity constants of 7.8×10^{-9} M for TF α , 1.1×10^{-9} M for aGPA, and 2.1×10^{-10} M for BG-H3. Such a high affinity for carbohydrate antigens is remarkable, especially since this lambdy originated from unstimulated naïve lamprey.

Our assays to define the carbohydrate specificity of VLRB.aGPA.23 indicated both BG-H3 and TF α -Serine structures are the main determinants recognized by this lambdy from among hundreds of structures that were presented on two glycan arrays. It is likely this lambdy is selective for an epitope that includes both elements of the TF α and blood group H structures. Extension of the TF α to the BG-H3 Fuc α 1-2 should be possible based on the crystal structure of the VLRB-BG-H2 trisaccharide complex.²¹ Assuming a similar glycan-binding site for VLRB.aGPA.23, it should be able to accommodate either TF α or fucosylated TF α , with about 40-fold tighter binding of the latter due to additional contacts with the VLR, as shown by SPR.

Lambdy Staining of Human Cancer Tissue Micro-arrays

We then tested the ability of VLRB.aGPA.23 to recognize tumor-associated glycans as they are presented in tissues, by immunohistochemistry (IHC) with human cancer tissue microarrays (TMA). This lambdy stained 14 out of 34 (41%) different types of adenocarcinomas and squamous cell carcinomas, including those of the bladder, breast, cervix, cheek, colon, esophagus, greater omentum, larynx, liver, lung, nasopharynx, nose, ovary, and tongue. We also tested by IHC three other anti-aGPA lambdies from this screen, but these produced either faint and diffuse staining patterns or stained normal tissues as well as tumors. For VLRB.aGPA.23, staining was undetectable in nearly all normal or benign tissues (Figure 5), except for one case of staining in a small portion of tumoradjacent normal lung tissue. In tumor tissues, various levels of membrane and cytoplasmic staining were visible in discrete patches of the tumors, thus indicating specific recognition of tumor antigens. We then focused on a cohort of tissue samples from 103 lung cancer patients

(Figure 6A), of which 27% stained positive with VLRB.aGPA.23, as ranked by digital scanning and quantitative analysis of the fractions of cells that stained positive. Among these patients, 88 were diagnosed with NSCLC, and those whose tumors stained positively with VLRB.aGPA.23 at medium to high intensities (27%) had a significantly poorer overall survival rate (Figure 6B).

Next we screened human cancer cell lines for expression of the VLRB.aGPA.23 antigen and used lysates from several positively staining cell lines for detection in a Western blot (Figure 7). The NSCLC cell lines (A545, H596, and H460), the head and neck squamous cell carcinoma cell line (HN6), and the breast cancer cell line (MCF7) all revealed a major reactive band of about 80 kDa, as well as minor bands of about 55 kDa, which were not detected in the normal lung cell line (HBE1).

We stained hundreds of human tissue samples with VLRB.aGPA.23 and observed high specificity for tumor antigens. This indicates that aberrantly glycosylated glycoproteins can be detected with highly selective lambodies. Importantly, the NSCLC patients whose lung tumors stained with this lambody at medium or high intensity had a significantly worse overall survival rate compared to those whose tumors stained negative or at low intensities, suggesting these abnormalities may be associated with the aggressive behaviors of the tumors. Lung cancer is the leading cause of cancer-related death in the United States,²² with an overall 5-year survival rate of only 15%. This lambody might be used to target tumors aberrantly expressing BG-H3 antigens, which are often observed together with other ABH antigens in oncogenesis of various organs.²³ For example, it has been reported that the normal colon mucosa expressed only BG-H1 antigen, whereas aberrant expression of BG-H2, with or without BG-H3/4 antigens, was observed in several cancers of the proximal and distal colon.²⁴ The same group reported that BG-H1 and BG-H3/4 antigens were expressed in the normal human lung only in apical surfaces of the bronchial epithelium.²⁵ However, the IgM mAb MBr1 used to detect BG-H3/4 antigens reacts mainly with the glycolipid globo H structure (Fuc α 1-2Gal β 1-3GalNac β 1-3Gal), which includes the BG-H4 antigen (Fuc α 1-2Gal β 1-3GalNac β), but it may not be able to detect the O-linked BG-H3 antigen. Lambody VLRB.aGPA.23 may turn out to be a useful tool in deciphering the role of its carbohydrate antigens in the pathology of cancer cells.

General Approach for Selection of Glycan-Binding VLRs

In order to evaluate the generality of our approach, we surveyed our VLR YSD library for binders of additional synthetic glycoconjugates and glycoproteins that display important biomedical glycotopes (Figure 1). These included monosaccharides such as mannose, the Tn pancarcinoma antigen (GalNac α), and *N*-glycolylneuraminic acid (Neu5Gc), the hydroxylated form of *N*-acetylneuraminic acid (Neu5Ac), which in humans is a dietary-derived sialic acid that can cause chronic inflammation and carcinomas.²⁶ Among the disaccharides we used TF α , and from the trisaccharides we used the Lewis antigens LeA [Gal β 1-3(Fuc α 1-4)GlcNac β] and LeX [Gal β 1-4(Fuc α 1-3)GlcNac β], whose uniquely sialylated forms are characteristic of most cancer cells.²⁷ As an example for polysaccharides we chose poly-Man9, a unique glycan of the HIV viral envelope, and gp120, its glycoprotein carrier.²⁸ Another target glycoprotein was aOSM, whose native form consists of 50% carbohydrates, mostly sialyl-Tn (94%) and TF α (4%). We then isolated clones that can bind these target glycotopes and sequenced batches of 8–24 clones per target. In each case 66–100% of the clones had unique sequences. As shown in Figure 8 for one representative from each screen, these clones bind their cognate ligands in a glycan-dependent manner. None of the clones reacted with the backbone spacer-PAA or with mannose except for the anti-Mannose clone. The anti-TF α clone was non-reactive to TF β , the anti-Neu5Gc only reacted with Neu5Gc, not with Neu5Ac, and the anti-LeA and anti-LeX were not cross-reactive. Alternating between poly-Man9 and gp120 as antigens in consecutive cycles of enrichment

enabled us to isolate clones with excellent affinity both for Man9 and gp120; for example, VLRB.gp120.4 (clone anti-gp120.4) had affinity constants of 6.2 nM for Man9 and 44.7 nM for gp120, determined by SPR.

All the glycan-binding VLR clones described here were isolated from a modest sized YSD library of 10^8 independent clones, which was constructed from lymphocyte RNA and genomic DNA of around 100 lamprey larvae and adults. These lamprey were collected in the wild and were not challenged with any of these glycoconjugates in order to produce an immune library.¹⁴ Nonetheless, by simple procedures we were able to isolate from this library good, as well as some excellent, binders for each of our biomedically important glycotope targets. For this survey we sampled and assayed selectivity of only small batches, 10–40 clones per target glycotope, from among populations of thousands positive clones that were sorted, which on the basis of our pilot sequencing samples should consist primarily of unique clones (60–100%). This indicates the library consists of large populations of highly diverse VLR clones that can bind each of these glycotopes and most likely many other important glycotopes. A high-throughput format of screening and validation will be required in order to harvest to exhaustion this highly useful resource of glycan-binding proteins.

On the surface of yeast, monomeric VLRs are displayed at densities that can enable cooperative binding by several VLRs of multivalent antigens, such as most glycoconjugates, generating stable complexes due to the high avidity. This is similar to the mode of binding of most high affinity lectins, which are typically multimeric proteins with multiple binding sites,²⁹ and pentameric IgM antibodies that are the common type of glycan-binding antibodies, with affinities typically in the range of 10^{-5} to 10^{-6} M. In contrast, the dimeric antibodies described here were either VLR-Fc fusion proteins, analogous to IgGs, or the compact VLR-GCN4 fusion proteins, and yet many of these could retain in solution their high affinity for glycoconjugates. For example, antibody VLRB.aGPA.23 binds BG-H3, aGPA, and TF α with dissociation constants of 0.2, 1, and 8 nM, respectively, and VLRB.gp120.4 had affinity constants of 6.2 nM for Man9 and 44.7 nM for gp120. These affinities rival those of most naturally occurring lectins, as well as conventional and recombinant antibodies. For comparison, a multimeric anti-TF α scFv had dissociation constants for aGPA of 88 and 220 nM, for the tetrameric and trimeric forms of the antibody, respectively;⁸ the antiviral lectin griffithsin³⁰ binds carbohydrates of gp120 with a dissociation constant of 8 nM; and a pentameric IgM antibody was isolated with an exceptionally high affinity constant of 200 pM for a glycopeptide epitope in aGPA.³¹ For antibody clones that are isolated with only moderate affinity for glycans, it should be possible to improve the binding by *in vitro* affinity maturation of the VLRs, as previously reported for VLR clones reactive to the protein lysozyme, with 100-fold improvement following a single cycle of random mutagenesis and 1,300-fold improvement following targeted mutagenesis.¹⁴

The ability to rapidly generate tailor-made antibodies to a variety of glycan antigens opens up numerous new opportunities for the field of glycobiology. Their selectivity for carbohydrates speaks to the rigorous conformational selection of the antibodies and hence their potential as an entirely new class of carbohydrate-binding proteins for glycomics research. In biomedical research, antibodies can be engineered as highly sensitive reagents to detect early stage cancers, which will provide better preclinical assessment at stages when medical intervention is most efficient. These can include noninvasive cancer diagnostics from body fluids, as well as *in vivo* tumor imaging. Furthermore, considering the biological roles of glycoproteins and their glycan structures in tumor cell invasion and metastasis, it is plausible that antibodies with their extraordinary high binding affinities may be able to interfere with functions of the glycoprotein carriers of tumor-associated carbohydrate structures and act as tumor targeting therapeutic agents that can save many human lives.

METHODS

Antigens

α -L-Fucose-PAA-biotin, Gal β 1-3GalNAc α -PAA-biotin (TF α), Gal β 1-3GalNAc α -PAA (TF α), Gal β 1-3GalNAc β -PAA-biotin (TF β), α -GalNAc-PAA-biotin (Tn), H(type 3)-PAA (BG-H3), LeA-PAA-biotin, LeX-PAA-biotin, α -D-Mannose-PAA-biotin, α -Neu5Ac-PAA-biotin, Neu5Gc α -PAA-biotin, and control spacer-PAA-biotin were purchased from Glycotect. YU2 3.1 core gp120 was a kind gift from Dr. G. Lewis, and poly-Man9-biotin from Dr. L-X. Wang (Institute of Human Virology, UMB). Human glycoporphin A, bovine submaxillary mucin, and ovine submaxillary mucin (Sigma) were desialylated by mild acid treatment, using 0.025 N H₂SO₄ in 0.85% NaCl, for 2 h at 80 °C, then neutralized with 1 M Tris-HCl pH 8.8, and dialyzed overnight against PBS pH 7.4 (QBI). Proteins were biotinylated with EZ-Link NHS-PEO₄-Biotinylation Kit (Pierce) at 1–3 mol biotin per mole protein.

YSD Library Screening

We screened a VLRB library of 1.2×10^8 independent clones.¹⁴ In order to increase the sensitivity of detection, yeast library cells were deglycosylated by treatment with Endo Hf, 5,000 units for each OD₆₀₀ of cells, in buffer G5 (NEB) supplemented with 0.1% Tween 20, shaking for 2 h at 30 °C. Antigen-binding clones were enriched initially by two rounds of MACS, then one or two rounds of FACS, using biotinylated antigens to label the cells. For the synthetic glycan screens, 500 nM of each glycan-PAA was used to label cells, both for the MACS and for sorting individual clones. For the anti-aGPA screen, cells labeled with 500 nM TF α -PAA were used for the first MACS, cells labeled with 500 nM aGPA for the second MACS, cells labeled with 500 nM TF α -PAA for the first FACS, and then cells labeled with 100 nM aGPA for sorting individual clones. For the anti-gp120 screen, cells labeled with 500 nM poly-Man9 were used for the first MACS, cells labeled with 100 nM gp120 for the second MACS, cells labeled with 20 nM poly-Man9 for the first FACS, and cells labeled with 500 nM gp120 for sorting individual clones. For the anti-aOSM screen, 5 μ g/mL aBSM was used to label cells in the first MACS, and 140 ng/mL aOSM for the second MACS and for sorting. Anti-biotin microbeads and MiniMACS separation unit were used for the MACS (Miltenyi). For staining and washing yeast cells, we used a buffer consisting of PBS pH 7.4, 0.5% BSA, 2 mM EDTA, and 0.1% Tween 20. For sorting, the VLRs were labeled with the desired antigen and 100 ng/mL of rat anti-FLAG (Stratagene). The cells were rotated for 25 min at RT and then placed on ice for 5 min, washed 3 times, and incubated with 1:1,000 dilutions of Alexa Fluor-488 conjugated donkey anti-rat IgG and SA-PE (Invitrogen) for 20 min on ice. Cells were washed 3 times with PBS, 0.1% BSA, and sorted using a FACSort equipped with a Cell Concentration Module (BD Biosciences). Data collection and analysis was done with CellQuest Pro software (BD Biosciences).

Yeast Secreted Lambdodies

The murine IgG2a Fc region from clone PS100053 (OriGene) was subcloned in plasmid pSCS2- α that was previously described,¹⁴ downstream from the *Sfi*I cloning sites, retaining the 6-His and FLAG tags. This resulted in plasmid p α -mIgG2a-Fc. VLR-Fc fusion proteins are homodimers of about 96–107 kDa. A compact homodimeric lambdoid format of about 56–67 kDa was constructed by replacement of the Fc region in plasmid p α -mIgG2a-Fc with the leucine zipper dimerization domain from the yeast GCN4 protein as described,³² immediately downstream from the IgG hinge, resulting in plasmid p α -GCN4.

Yeast strain YVH10 was used for secretion of lambdodies, cultured for 96 h at 30 °C in BYPDG, pH 6.7 (2% Bacto Peptone, 1% Bacto Yeast Extract, 44 mM Na₂HPO₄·7H₂O, 56 mM NaH₂PO₄, 2% glucose, 2% galactose, 0.05% Tween 20, and 100 μ g/mL G418), with

fresh 2–4% galactose added daily.¹⁴ Lambodies were purified from secretion supernatants using Ni-NTA agarose (QIAGEN). Protein concentrations were determined using a Micro BCA Protein Assay (Pierce). Typical yields of lambodies expressed in p α -mIgG2a-Fc were 2–5 mg/L, and 5–10 mg/L from p α -GCN4 clones, and these proteins are stable for at least 6 months when stored at 4 °C. In native PAGE the VLR-GCN4 fusion proteins migrate as sharp bands, indicating no oligomerization or aggregation of the proteins (not shown)

Glycan Array

For glycan array profiling we used the biotinylated fusion proteins VLRB.aGPA.23-GCN4 and VLRB.2D-GCN4, a lysozyme binding clone¹⁴ that served as a control. These lambodies were biotinylated via Maleimide-PEG₂-Biotin (Pierce), yielding about 2 mol biotin per mole protein. For the CFG printed array Version 5.0 was screened at three concentrations of the lambody: 2, 20, and 200 μ g/mL, following the CFG protocol that is available at <http://www.functionalglycomics.org>.

For the neoglycoprotein microarray^{17,33} biotinylated VLRB.aG-PA.23-GCN4 was screened at three concentrations: 1, 10, and 100 μ g/mL. Briefly, arrays were blocked with 3% BSA in PBS at 4 °C overnight, and then the lambody was incubated on the array in fresh 1% BSA in PBST at 25 °C for 2 h. Slides were washed with PBST, and then bound VLRB.aGPA.23 was detected with Cy3-labeled streptavidin (Invitrogen) at a 1:500 dilution in fresh 1% BSA in PBS, at 25 °C for 1 h. The glycan microarrays were scanned using GenePix Pro (Molecular Devices) at a PMT setting of 520, and data for each array component were averaged over 2 spots. A complete list of array components on each array can be found in Supplementary Spreadsheet 1A and 1B.

SPR Analysis

All binding experiments were performed using Biacore T200 (GE Healthcare). The running and sample buffer was HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Polysorbate 20). All measurements were performed at 25 °C. Anti-His antibody (His capture kit, GE Healthcare) was immobilized by amine coupling to Sensor Chip CM5. VLRB.aGPA.23-GCN4 and VLRB.gp120.4-GCN4 were diluted to 10 μ g/mL and injected in each cycle at 10 μ L/min for 30 s, to capture approximately 300 RU. The antigens aGPA, TF α -PAA, and BG-H3-PAA were serially diluted in HBS-EP+ from 40 nM to 156 pM; poly-Man₉ was serially diluted from 250 to 1 nM; and the gp120 was serially diluted from 500 to 31.2 nM. The antigens were injected at a flow rate of 30 μ L/min. Regeneration of the anti-His surface was accomplished using a 60 s injection of 10 mM glycine-HCl, pH 1.5. The reference subtracted SPR binding curves were blank subtracted, and curve fitting was performed with a 1:1 model to obtain kinetic parameters using the Biacore T200 Evaluation software.

Immunohistochemistry

TMA's consisting of formalin-fixed and paraffin-embedded human tissue cores were purchased from US Biomax, Pantomics, and IMGENEX. TMA's IMH-305 and IMH-358 (IMGENEX) were available with lung cancer patient survival data. Standard IHC protocol was followed. Briefly, TMA's were deparaffinized by several changes of xylene for a total of 30 min. The slides were then rehydrated in gradual ethanol, before immersion in PBS, pH 7.4. Endogenous peroxidase was quenched by 20 min incubation in 3% H₂O₂ in methanol. Antigen retrieval was performed by immersion in 1 mM EDTA, pH 8.0, in a water bath for 10 min at 96 °C. Slides were blocked with 10% normal horse serum (Invitrogen), 1% BSA (Pierce), for 1 h at RT. Lambody VLRB.aGPA.23-mFc was diluted to 5 μ g/mL in PBS, pH 7.4, 1% BSA, 0.005% Tween 20, 0.01% Triton X-100 and incubated overnight at 4 °C. After washing in PBST, the lambodyantigen complexes were detected with ImmPRESS

anti-mouse IgG-HRP and ImmPACT DAB peroxidase substrate and counter-stained with hematoxylin (Vector Laboratories).

Stained TMAs were scanned and scored using Aperio ScanScope XT instrument and the image analysis platform (Vista). Digital images of stained tissue cores were analyzed by the Aperio Membrane v9 algorithm that reports for each core the percentage of positive membrane staining and average intensity in 4 categories: 0 (% negative cells), 1 (% low intensity), 2 (% medium intensity), and 3 (% high intensity). The arbitrary threshold for negative staining was set on the basis of values of normal lung tissue. Scores of samples from categories 0 and 1 were pooled and arbitrary ranked as Negative staining, and scores of samples from categories 2 and 3 were pooled and arbitrary ranked as Positive staining. The association between positive staining with VLRB.aGPA.23 and patient clinical outcome was estimated by the method of Kaplan–Meier. The Mantel-Cox log-rank test was used to calculate univariate correlation. The clinical diagnosis of samples was according to TMA datasheets provided by IMGENEX.

Western Blot

Cell lines A549, H596, H460, and MCF7 were purchased from ATCC, HN6 was a kind gift from Dr. J.S. Gutkind (National Institute of Dental and Craniofacial Research), and HBE1 was from Dr. J. Minna (University of Texas Southwestern Medical Center). Cancer cell lines were grown in DMEM supplemented with 10% FBS, and HBE1 cells were grown in keratinocyte-serum free medium supplemented with EGF1-53 and bovine pituitary extract (Invitrogen). Protein content from whole cell lysates were quantified using BCA assay (Pierce). Per lane, 25 μ g of protein was fractionated under non-reducing conditions in NuPAGE 10% Bis-Tris gel (Invitrogen) and transferred onto a PVDF membrane, using the iBlot gel transfer (Invitrogen). Membranes were blocked in TBST, 5% BSA, for 1 h then incubated overnight at 4 °C either with 0.5 μ g/mL of VLRB.aGPA.23-mFC in TBST, 1%BSA, or with a mouse anti- β -actin mAb (Sigma). After washing, the complexes were detected using anti-mouse-HRP and enhanced chemiluminescence reagent (GE Healthcare). BenchMarker protein ladder (Invitrogen) served to estimate molecular weights.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structures used for selection of antiglycan lambdoids. (1) Schematic depiction of polyacrylamide (PAA) linear polymer with multiple TF α structures. Covalent attachments to the PAA backbone are formed from functional linkers (wavy lines) via amide bonds. Other sugars used were (2) mannose-PAA, (3) fucose-PAA, (4) BG-H3-PAA, (5) GalNAc α -PAA, (6) Neu5Ac α -PAA, (7) Neu5Gc α -PAA, (8) LeA-PAA, (9) LeX-PAA, and (10) poly-Man9. The blood group H3 trisaccharide is shown in a box with the H-disaccharide shaded in light blue.

Figure 2.

Glycoconjugate selectivity of two anti-aGPA clones, VLRB.aGPA.11 and 23. Flow cytometry dot-plot analysis of ligand binding intensities, presented as the Y-mean fluorescence of ligand for aGPA, native GPA, TF α -PAA, TF β -PAA, fucose-PAA, and mannose-PAA, which were used to label at the indicated concentrations (Y mean < 3 indicates no binding). All antigens were biotinylated and were detected with phycoerythrin-labeled streptavidin (SA-PE). The level of VLR surface display was detected by labeling with a rat anti-FLAG mAb followed by anti-rat Alexa 488 antibodies.

Figure 3. Glycan binding profile of VLRB.aGPA.23-GCN4. The main four glycan structures recognized are highlighted. Results on the CFG array V5.0, consisting of 611 printed glycans, reacted with 2 $\mu\text{g/mL}$ lambdoid.

Figure 4.

Kinetic analysis of binding glycoconjugates by VLRB.aG-PA.23-GCN4. Representative SPR sensorgrams for interaction of the immobilized antibody with TF α -PAA, aGPA, and BG-H3-PAA are shown for concentrations of 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, and 40 nM. Reference subtracted and blank subtracted binding data were fitted with a 1:1 Langmuir binding model to determine the association and dissociation constants. The equilibrium dissociation constants were calculated from these values. The statistics for χ^2 and U -values are shown, and all T -values for k_a , k_d , and R_{max} were >100 .

Figure 5.

Lambdoid staining of human normal and cancer tissues. (A) Normal lung (tumor adjacent), (B) lung large cell carcinoma, (C, D) lung squamous cell carcinoma, (E) normal colon (tumor adjacent), (F) colon adenocarcinoma, (G) normal prostate, and (H) prostate adenocarcinoma. Images were scanned at 20x magnification. Tissue microarray slides were incubated with 5 $\mu\text{g/mL}$ of VLRB.aGPA.23-mFc, and the immune complexes were detected with anti-mouse IgG-HRP and DAB substrate (brown) and then counterstained with hematoxylin (blue).

Figure 6.

Detection of human lung cancers with VLRB.aGPA.23-mFc. (A) Summary of staining lung tumors and adjacent normal lung tissues ($n = 112$). Samples were ranked on the basis of image analysis scores for fractions of positively staining cells per tissue core. Positive staining – pooled medium and high intensity scores per tissue; Negative staining – pooled unstained and low intensity scores per tissue. Cases of non-small cell lung cancers (NSCLC; $n = 88$) are shaded yellow. (B) Relationship between overall survival rate of NSCLC patients and staining with VLRB.aGPA.23. Kaplan–Meier curves for samples that stained positive ($n = 24$), compared to negative staining ($n = 64$). Inset: p value calculated using the Mantel–Cox log-rank χ^2 test.

Figure 7.

Detection of VLRB.aGAP.23 antigen expression in cancer cell lines. Whole cell protein lysates were fractionated under non-reducing conditions from the non-small cell lung cancer (NSCLC) cell lines A549, H596, and H460; the head and neck squamous cell carcinoma HN6; the breast cancer MCF7; and the normal bronchial epithelium cell line HBE1. After transfer, the membrane was stained with VLRB.aGAP.23-mFc or with anti- β -actin as a loading control.

Figure 8.

Survey of VLR clones that can selectively bind monosaccharides, disaccharides, trisaccharides, polysaccharides, and glycoproteins. Flow cytometry dot-plot analysis of ligand binding intensities, presented as the Y-mean fluorescence of ligand, for a representative clone from each screen (Y mean < 3 indicates no binding). (A) Clone anti-Mannose.5 labeled with the indicated concentrations of mannose-PAA (top plot) and the control spacer-PAA (bottom plot). (B) Anti-GalNAc.2 labeled with GalNAcα-PAA and mannose-PAA. (C) Anti-Neu5Gc.8 labeled with Neu5Gcα-PAA and Neu5Acα-PAA. (D) Anti-TFα.1 labeled with TFα-PAA and TFβ-PAA. (E) Anti-LeA.3 labeled with LeA-PAA and LeX-PAA. (F) Anti-LeX.11 labeled with LeX-PAA and LeA-PAA. (G) Anti-gp120.4 (clone VLRB.gp120.4) labeled with poly-Man9 and gp120. (H) Anti-OSM.2 labeled with aOSM and TFα-PAA. Biotinylated antigens were detected with SA-PE, and the level of VLR surface display was detected by labeling with rat anti-FLAG mAb followed by anti-rat Alexa 488 antibodies.