Multivalent Binding of Carbohydrates by the Human α-Defensin, HD5[1]

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Four of the six human α-defensins (human neutrophil peptides 1–3 and human α-defensin 5; HD5) have a lectin-like ability to bind glycosylated proteins. Using HD5 as a model, we applied surface plasmon resonance techniques to gain insights into this property. HD5 bound natural glycoproteins > neoglycoproteins based on BSA > nonglycosylated BSA ≫ free sugars. The affinity of HD5 for simple sugars covalently bound to BSA was orders of magnitude greater than its affinity for the same sugars in solution. The affinity of HD5 for protein-bound carbohydrates resulted from multivalent interactions which may also involve noncarbohydrate residues of the proteins. HD5 showed concentration-dependent self-association that began at submicromolar concentrations and proceeded to dimer and tetramer formation at concentrations below 5 μM. The (R9A, R28A) and (R13A, R32A) analogs of HD5 showed greatly reduced self-association as well as minimal binding to BSA and to BSA-affixed sugars. From this and other evidence, we conclude that the extensive binding of HD5 to (neo)glycoproteins results from multivalent nonspecific interactions of individual HD5 molecules with carbohydrate and noncarbohydrate moieties of the target molecule and that the primary binding events are magnified and enhanced by subsequent in situ assembly and oligomerization of HD5. Self-association and multivalent binding may play integral roles in the ability of HD5 to protect against infections caused by viruses and other infectious agents. ***The Journal of Immunology,*** **2009, 183: 480–490.**

Defensins are small, 2.0- to 4.5-kDa peptides that contribute to host defense against microbial (1–3) and viral (4–7) infections by interacting with the pathogen directly, or in concert with other components of the immune system (8, 9). The three subfamilies of defensins (α, β, and θ) found in mammals evolved from a common ancestral gene (10, 11) whose β-defensin progeny extend back to bony fishes (12), if not beyond (13). All of these defensin peptides contain six cysteines, have three intramolecular disulfide bonds, and are cationic. To date, α-defensin peptides have been found only in mammals (14) and θ-defensins have been identified only in certain nonhuman primates (11, 15, 16). α-Defensins have a largely β-sheet structure composed of 29- to 35-aa residues. θ-Defensins are cyclic and have only 18 residues. Uniquely, their cyclic backbone is formed by posttranslational ligation of two precursor peptides (11, 17).

Given the involvement of defensins in antimicrobial and antiviral host defense, it can be surmised that they possess an ability to distinguish self from nonself targets. Their preferential binding to microbial surfaces and membranes is attributable, in part, to the net positive charge of defensins and the fact that microbial surfaces and membranes are typically enriched in anionic moieties such as lipoteichoic acid, lipid A, and anionic phospholipids (18). Defensins also bind to and/or inactivate enveloped viruses, the membranes of which are host cell derived. In such instances, carbohydrate epitopes of glycan chains afford likely biorecognition sites (19, 20). The carbohydrate-binding ability of θ-defensins has been documented and examined with respect to their antiviral properties (21, 22) and also because θ-defensins are among the smallest known lectins (23–25). Humans express six different α-defensins. Four were first discovered in neutrophils, and are called human neutrophil peptides (HNP)[3] 1–4 (16, 26, 27). The other two human α-defensins (HD), HD5 and HD6, are expressed primarily by small intestinal Paneth cells (28) and in regions of the female urogenital tract (26, 29). Murine Paneth cell α-defensins, also called cryptdins, protect intestinal crypts from microbial incursions and influence the resident microbial flora of the small intestine (30). Human HD5 deficiency has been linked to the pathogenesis of certain diarrheal infections (31) and to that of ileal Crohn's disease (32–34), although the latter assertions have been challenged (35).

In recent studies, HNPs 1–3 and HD5 proved to be potent antagonists of infection by human papillomaviruses, the primary causes of cervical cancer (36). HD5 was particularly active against sexually transmitted papillomaviruses, with IC_{50} values in the high nanogram per milliliter range. HD5 did not block virion binding or internalization, instead acting by preventing the escape of virions from their endocytic vesicles (36). HD5 inhibits adenovirus infections by binding to the virus capsid and preventing its subsequent

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[3] Abbreviations used in this paper: HNP, human neutrophil peptide; BSA-β-D-Glc-NAc, a neoglycoprotein containing β-D-*N*-acetylglucosamine residues covalently bound to BSA; CM, carboxymethylated; $FO_{0.5}$, the peptide concentration that binds, on average, one-half of the sugar residues on a neoglycoprotein; gD1, glycoprotein D of herpes simplex type 1; HD, human defensin; $IC_{0.5}$, concentration of an inhibitor that reduces binding of 1 μg/ml HD5 to the binding level shown by 0.5 μg/ml (in this report); *m/z*, mass divided by charge; MR, molar ratio; RU, response (or resonance) units; SPR, surface plasmon resonance.

uncoating, an event the virus requires to escape the endosomal compartment and enter the cytosol (37). HD5 and HNP1 also inhibit infection by BK polyoma virus, a cause of interstitial nephritis in immunosuppressed renal transplant recipients, evidently acting by binding to the virions and agglutinating them (38).

In this article a systematic approach is used to detect carbohydrate binding with respect to sugar specificity and density of epitope presentation. Toward this end, a panel of chemically glycosylated molecules was prepared, using BSA as carrier. These neoglycoproteins have been useful sensors for lectin sites in previous studies with isolated proteins and different cell systems (39–43). Several natural glycoproteins were also tested to characterize the carbohydrate-binding properties of HD5. Our detailed examination of sugar binding by HD5 demonstrates that multivalent binding and the self-association of the peptide play important roles in this process. Although we did not study viruses directly, our findings may explain how HD5 can cross-link intact viruses to cause agglutination or elements of a viral capsid to prevent viral uncoating.

Materials and Methods

Glycoproteins

Recombinant glycosylated glycoprotein D (gD1) from HSV-1, McIntyre strain) was purchased from Fitzgerald Industries. It contained aa 21–339 of the ectodomain and had an estimated nonglycosylated mass of 35,238 Da. HIV-1_{BAL} gp120, immunoaffinity purified from infected HEK 293 cells, was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Its molecular mass was ~116,000 by SDS-PAGE, and its nonglycosylated mass was 54,157. HIV-1_{LAV} gp120 (Protein Sciences) was a full-length glycosylated envelope protein produced in insect cells by a baculovirus system.

Neoglycoproteins

Chemically glycosylated BSA-based neoglycoproteins were prepared by two standard procedures. The neoglycoproteins in set 1 were made by reacting diazonium salts derived from *p*-aminophenyl glycosides obtained by the catalytic reduction of corresponding *p*-nitrophenyl glycosides (44). The neoglycoproteins in set 2 were prepared by conjugating *p*-isothiocyanatophenyl glycosides made by treating *p*-aminophenyl glycosides with thiophosgene to the carbohydrate-free carrier protein (44). The reagents used to carry out these procedures were obtained from Sigma-Aldrich. Mass analyses were done on a Voyager System-4299 MALDI-TOF instrument (Applied Biosystems), using a matrix of sinapinic acid. The mean extent of glycosylation was estimated by first subtracting the peak mass of unmodified BSA (66, 650) from the peak mass of the neoglycoprotein and then dividing this difference by the molecular mass of the carbohydrate plus that of the linker, which was 88 mass units for the neoglycoproteins in group 1 and 104 for those in group 2. Diazonium-derivatized carbohydrates will attach primarily to tyrosyl, histidyl, and lysyl residues, and isothiocyanato groups should attach almost exclusively to amino groups, especially those of lysine (44).

Surface plasmon resonance (SPR)

CM5 chips have four compartments (flow cells) with surfaces that consist of a carboxymethylated (CM) dextran matrix covalently attached to a thin gold film. When working with neoglycoproteins, we reserved one CM5 compartment to measure background binding to the surface matrix, attached carbohydrate-free BSA to another, and attached neoglycoproteins to the remaining two. When working with natural glycoproteins, only the CM-dextran compartment was used as a background control. Proteins and peptides were immobilized on the CM5 chips by amine coupling per the manufacturer's instructions, using reagents (*N*-hydroxysuccinimide, 1-ethyl-3-(3-aminopropyl)carbodiimide hydrochloride, and ethanolamine hydrochloride) purchased from Biacore. The analyte buffer, HBS-EP, contained 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20, and 0.01 M HEPES (pH 7.4). Binding was monitored at 1-s intervals for 5 min with an analyte flow rate of 50 μl/min. Dissociation was monitored at 1-s intervals for 2–4 min. The sensor chips were regenerated by washing them with 10 mM HCl, and (if needed) 10 mM NaOH. Data analysis was performed using BIAevaluation 4.1 software from BiaCore, and Sigma Plot 9 and Sigma Stat 3.0 software from Systat.

Results of SPR experiments are expressed in resonance units (RU), which sense changes in the refractive index close to the metal surface of a biosensor. According to Biacore and other sources, 1 RU is equivalent to 1 pg of protein per mm^2 of sensor surface (45–47). Because the sensor surface of a CM5 chip is close to 1 mm^2, the target density (2000 RU), we selected for the neoglycoprotein-presenting biosensors corresponds to ~2 ng of BSA/mm^2. In actuality, the many biosensors used in this study contained, on average, 1878.2 ± 60.4 RU (mean ± SEM; $n = 29$) and ranged from 1392 to 2617 RU, necessitating certain corrections that are described in the text.

Compensating for background binding to BSA

We assumed that background binding of any given concentration of HD5 to carbohydrate-free BSA immobilized on a CM5 biosensor was directly proportional to the BSA density, in RU. Data supporting this assumption is provided in the text. Accordingly, if BSA compartment of the biosensor had a ligand density of 1600 RU and the neoglycoprotein compartment of the biosensor had a ligand density of 1950 RU, we multiplied the RU bound to the BSA compartment by 1.219 (1950/1600) before subtracting it from the RU bound to the neoglycoprotein compartment to obtain the net binding attributable to the bound carbohydrate.

Molar ratio and fractional occupancy

Molar ratio (MR; Equation 1) signifies the number of molecules of HD5 bound per molecule of neoglycoprotein. MR_{sa} (Equation 2) signifies the molar ratio, corrected for the self-association of HD5. Fractional occupancy (FO) signifies the number of HD5 molecules, corrected for self-association, per attached carbohydrate molecule. In Equation 3, the self-association factor, s, corresponds to the molar ratio shown in Fig. 5.

$$MR = (ab)/(cd) \quad (1)$$

$$MR_{sa} = MR/(1 + s) \quad (2)$$

$$FO = MR_{sa}/C_{mean} \quad (3)$$

In Equation 1, a = net (i.e., background-corrected) binding in RU at 300 s; b = mean neoglycoprotein mass; c = mass of the analyte (HD5 or HD5 analog); d = ligand density (RU), of the neoglycoprotein immobilized on the biosensor. In Equation 3, C_{mean} represents the mean number of carbohydrate residues on each neoglycoprotein, determined by MALDI-MS analysis as described in the text. Finally, the $FO_{0.5}$ indicates the concentration of HD5 responsible for the net binding of 0.5 HD5 molecules per carbohydrate molecule on the neoglycoprotein. The $FO_{0.5}$ is determined graphically from Equation 3, as illustrated in the text below.

Results

Binding of HD5 to natural glycoproteins

Fig. 1 shows the binding of HD5 to three glycoproteins: fetuin; $gp120_{LAV/IIIB}$; and gD1 of HSV-1. At 1 μg/ml, HD5 bound each glycoprotein extensively but showed little binding to nonglycosylated BSA. Increasing the HD5 concentration to 10 μg/ml did not overcome this differential. To estimate the affinity of HD5's binding, we used BIAevalution 4.1 software, assumed 1:1 binding, and tested six concentrations of HD5 from 50 to 300 ng/ml. In two experiments, done on different days, the mean K_d values ± SD were 14.4 ± 4.9 and 15.7 ± 14.0 nM for fetuin, 23.5 ± 14.0 and 26.0 ± 12.9 nM for gD of HSV-1, and 24.5 ± 10.8 and 23.0 ± 25.7 nM for gp120 from HIV-$1_{LAV/IIIB}$.

We also evaluated HD5 binding by measuring the ability of solution phase glycoproteins to inhibit binding of 1 μg/ml HD5 to an appropriate biosensor. In Fig. 2*a*, the standard curve shows the binding of HD5 to a CM5 biosensor containing immobilized fetuin in the absence of any competitor. The open circles show that adding different concentrations of fetuin to 1 μg/ml (279 nM) of HD5 in the analyte solution caused a concentration-dependent inhibition of HD5 binding to the biosensor.

Fetuin, an acidic (pI 3.3) protein with sialic acids in the bi- and triantennary complex-type *N*-glycans and core 1 *O*-glycans, has a mass of 48,400 Da (48). The horizontal and vertical reference lines show that 9 μg/ml fetuin (186 nM) reduced binding of 1 μg/ml

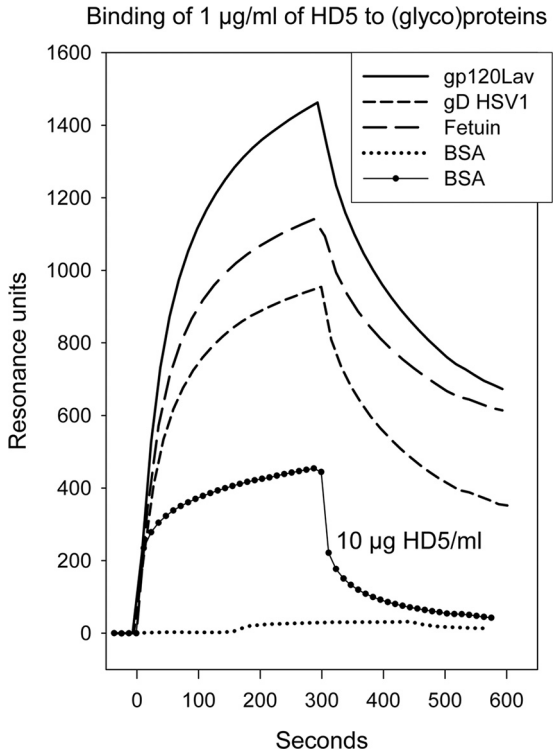

FIGURE 1. Binding of HD5 to selected (glyco)proteins. The biosensor chips presented 3917 RU of BSA, 3911 RU of bovine fetuin, 4296 RU of glycoprotein D (gD) of HSV-1, and 5655 RU of gp120 from $HIV1_{LAV}$. Except where noted, the concentration of HD5 was 1 μg/ml.

HD5 to the level seen with 0.5 μg/ml HD5 alone. The concentration that reduces binding of 1 μg/ml HD5 to that of 0.5 μg/ml will henceforth be called the $IC_{0.5}$.Applied in conjunction with the Law of Mass Action (Equation 1, below), the $IC_{0.5}$ allows the affinity of binding to be estimated.

$$[\text{HD5}][\text{fetuin}]/[\text{HD5-fetuin}] = K_d \quad (4)$$

To illustrate, we will apply Equation 4 to the experiment shown in Fig. 2*a*. Because [HD5] and [HD5-fetuin] both equal 139.5 nM, [fetuin] equals 186 − 139.5 nM, or 46.5 nM. This estimate of the K_d, 46.5 nM, is almost identical with the K_d value (41.4 nM) obtained by directly monitoring binding of 1 μg/ml HD5 to immobilized fetuin and using BIAevalution 4.1 software and the assumption of 1:1 binding to analyze the binding curve.

In competition assays, the molecule in solution need not be the same as the molecule on the biosensor. Fig. 2*b* shows a competition experiment using the same fetuin-containing biosensor but with $gp120_{LAV}$ as the competing analyte. Nonglycosylated $gp120_{BAL}$ has a mass of 54,157 and a pI of ~8.43. In Fig. 2*b*, 13.4 μg of gp120/ml (~247.4 nM) reduced HD5 binding to the $IC_{0.5}$ level. Applying Equation 4 and assuming 1:1 binding, this corresponds to a K_d of ~108 nM. We also tested glycoprotein D of HSV-1 in the same manner. This viral glycoprotein contains three sites for *N*-glycosylation (49), and has a mass of 35,238 (exclusive of its glycans), and a pI of 7.20. It required 7.3 μg/ml gD1 (207 nM) to reduce binding of 1 μg/ml of HD5 to the $IC_{0.5}$ level. Applying this finding to Equation 4 gave an estimated K_d of ~67.5 nM (data not shown). Whereas fetuin and gD1 contain only 3 *N*-linked glycosylation sites each, $gp120_{BAL}$ has as many as 24, including 11 high-mannose or hybrid-type glycans and 13 complex-type glycans (50). Consequently, over one-half of the mass of gp120 is composed of carbohydrate (51, 52).

HD5 also bound to BSA but with much lower affinity. In the competition assay, 742 μg/ml nonglycosylated BSA reduced HD5 binding to the $IC_{0.5}$ level, corresponding to a K_d of ~11.2 μM. Having established that HD5 binds with high affinity to natural glycoproteins, we proceeded to dissect sugar specificity with a panel of neoglycoproteins carrying sugar units found in animal and bacterial glycans to characterize the selectivity of the carbohydrate binding of HD5 at the level of mono- and disaccharides. Although this was simple enough in concept, it proved to be difficult in practice due to unanticipated features of the binding behavior of HD5 that are described below. We will start with the simplest of these, background binding.

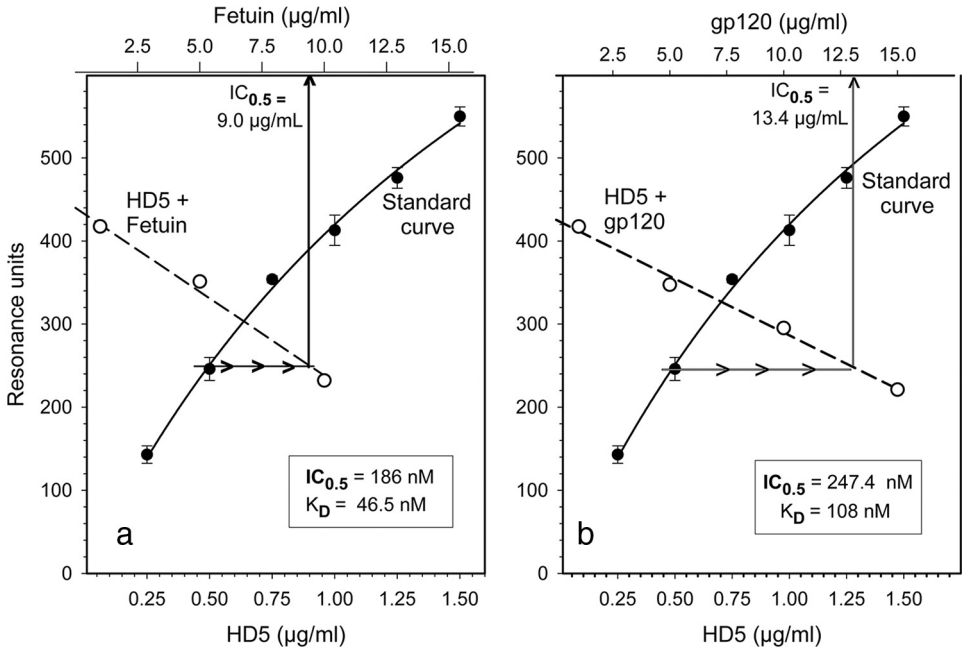

FIGURE 2. Competition assay. In both panels, the solid circles show binding, in RU, to a CM5 biosensor presenting 1873 RU of immobilized fetuin. Data are means ± SEM from three replicate experiments performed on consecutive days. ○, Binding of the analyte, which contained 1 μg/ml HD5 plus various concentrations of fetuin (*a*) or $gp120_{BAL}$ (*b*). The dashed line represents a least-mean-squares fit to the data. Horizontal line plus arrowheads, binding by 0.5 μg/ml HD5. A vertical line arises from the intersection of this horizontal line and the standard curve. This vertical line crosses the *x*-axis at the $IC_{0.5}$ of fetuin (*a*) and $gp120_{BAL}$ (*b*). The K_d can be calculated from the $IC_{0.5}$, as described in the text.

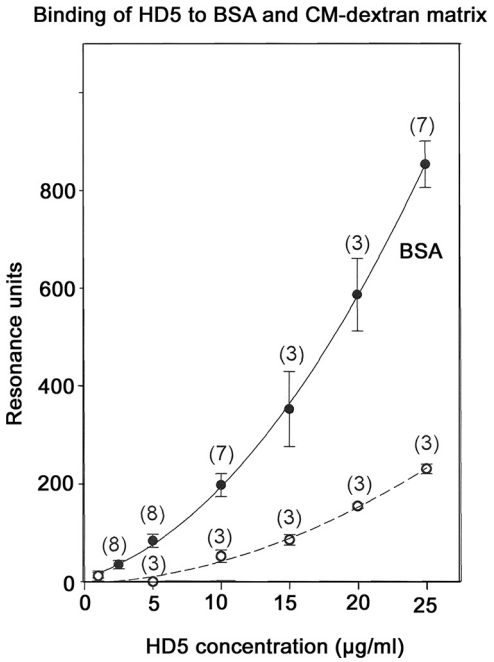

FIGURE 3. Background binding. ●, Binding of HD5 to immobilized BSA; ○, its binding to the CM-dextran matrix. Data points and error bars represent means ± SEM. Numbers in parentheses, number of observations. The biosensors presented 1778 ± 73.6 RU of immobilized BSA (mean ± SEM; range, 1532–2015 RU).

Background binding

Our intended biosensors would be neoglycoproteins covalently bound to the CM-dextran matrix of Biacore CM5 biosensor chips. Consequently, it was necessary to examine the background binding of HD5 to each of these. Fig. 3 shows that HD5 bound both the matrix and BSA, and that its binding to BSA was ~4-fold higher than its binding to the matrix. Whereas background binding to the matrix was easily handled by the instrument's software, background binding of HD5 to BSA was more problematic because we could not produce biosensors whose BSA content (in RU) precisely matched the RU of the BSA-based neoglycoproteins affixed to the other biosensor surfaces on the same CM5 chip.

Individual CM5 biosensor chips contain four flow cells, each having a biosensor. Accordingly, we prepared a flow cell with sensors containing three different amounts of bound BSA to see how ligand density influenced HD5 binding (Fig. 4). Scaling was almost perfectly linear over the full RU range of BSA encompassed in our experiments; therefore, compensating for binding to the protein portion of the neoglycoproteins was straightforward.

Self-association of HD5

NMR studies and x-ray crystallography have shown that all six human α-defensins can form dimers or higher-order oligomers (53–56). Because such studies used much higher defensin concentrations than the micromolar levels used in our SPR experiments, we needed to determine whether similar HD5 self-association also occurred under our experimental conditions. To do so, we attached HD5 to a Biacore CM5 chip by standard amino coupling and used this sensor to examine HD5-HD5 binding. Because the only free amino group of HD5 is located at its N terminus, the orientation of the peptide on the biosensor was both uniform and determined. The left ordinate scale of Fig. 5 shows binding, in RU, at seven concentrations of HD5. The right ordinate scale shows this binding as a MR, to indicate how many HD5 molecules had bound per molecule of immobilized HD5. In this experiment, analyte containing ~8.6 μg of HD5 per ml (2.4 μM) had an MR of 0.5. Using another CM5 chip that contained 799.1 RU of bound HD5, we found a MR of 0.5 at 7.68 μg of HD5/ml (2.14 μM). Averaging these results, we estimated that the affinity (K_d) of HD5 for another HD5 molecule is ~2.27 μM. Consequently, it would be necessary to consider self-association in our binding studies, especially at high HD5 concentrations.

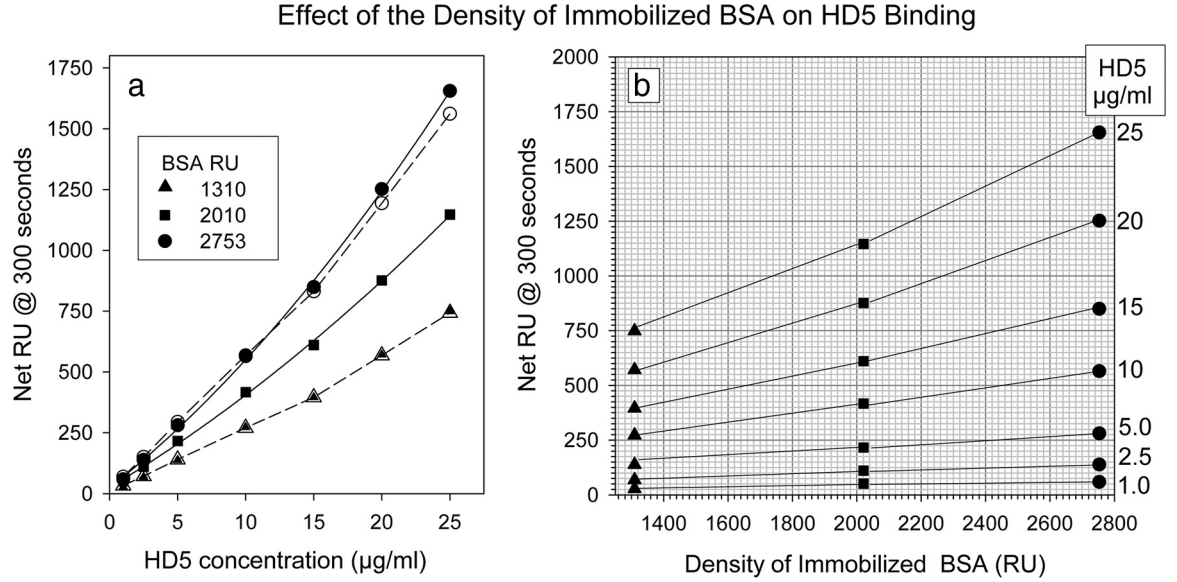

FIGURE 4. *a*, Analyte containing various concentrations of HD5 (from 1 to 25 μg/ml) flowed sequentially for 5 min over CM5 biosensors presenting unmodified BSA at three densities, 1310, 2010, and 2753 RU. The peak binding at each time is shown in solid symbols. ○ and △, Predicted RU, based on the values observed with the 2010 RU-biosensor, as explained in the text. The lines that connect the solid symbols were fit to a polynomial function, $y = y_0 + ax + bx^2$, values for the middle curve (■) of which were: $y_0 = 25.62$, $a = 33.53$, and $b = 0.4473$. The predicted values (○ and △) are connected by an interrupted line. *b*, The same observed data (solid symbols) as *a*, but in a different format that can also be used to scale background binding to BSA if the simpler procedure exemplified in *a* is not used.

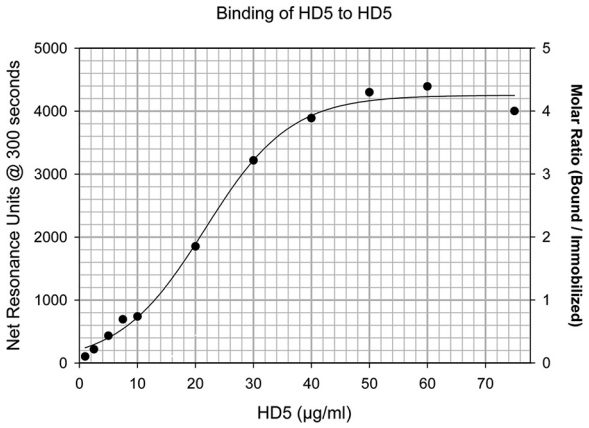

FIGURE 5. Self-binding of HD5. HD5 was immobilized on a CM5 biosensor chip to a density of 991 RU. Analyte solutions that contained 1–75 μg of HD5 per ml were introduced into the flow cell chambers, and binding was allowed to proceed for 5 min (300 s). Data points show net binding to the biosensor, in RU, at the end of this period. Net binding signifies that the values were corrected for background binding to the matrix of the CM5 biosensor chip. Binding was linear with concentration up to 7.5 μg/ml, but thereafter the binding curve was sigmoidal. The molar ratio (right ordinate) was derived by dividing net binding (left ordinate) scale by 991.1, the density of HD5 on the biosensor.

Characteristics of the neoglycoproteins

We used two sets of neoglycoproteins in these studies, each made by different chemical strategies starting from *p*-aminophenylglycosides (44). When examined by MALDI-MS, the *m/z* spectra of these neoglycoproteins showed symmetrical peaks that were shifted from that of unmodified BSA by the added mass of the attached carbohydrates (Fig. 6). After examining different ways of calculating the mean mass increase, we chose the simplest one, i.e., the difference between the peak masses. Among 11 neoglycoproteins in set 1, the mean number of carbohydrate residues per BSA ranged from 0.67 (BSA-maltose) to 5.45 (BSA-β-D-GlcNAc), with a mean $\pm$ SD of 2.28 $\pm$ 1.56 sugars per BSA. The seven neoglycoproteins in set 2 contained increased number of carbohydrate residues per BSA carrier (mean $\pm$ SD, 9.32 $\pm$ 0.92) and had a rather uniform mean carbohydrate content, ranging from 7.6 to 10.3 sugars per carrier.

Binding of HD5 to sugars attached to BSA

Thus far, we have shown that the RU in binding curves can result from binding of HD5 to the matrix of the sensor, to unmodified BSA, and to HD5 molecules bound to either the matrix or BSA. Fig. 7 shows an experiment done with a sensor chip with a surface that contained 1893 RU of immobilized BSA with α-glucose units, a set 2 neoglycoprotein displaying a mean of 7.6 glucose molecules per BSA. Fig. 7*a* shows the binding, in RU, specifically attributable to the presence of the sugars. To obtain these values, the observed RU bound to BSA was first adjusted to compensate for its lower ligand density (1532 RU) and then subtracted from the RU bound to the neoglycoprotein. The open circles in Fig. 7*b* show RU values at the end of the 300-s binding period; the solid circles represent the same data points corrected for HD5 self-association. The area between the two curves reflects the magnitude of self-association. From the corrected curve (●), the $FO_{0.5}$ equals ~15.5 μg HD5 per ml (4.32 μM).

Table I shows $FO_{0.5}$ values obtained from similar analyses that were done with nine types of neoglycoproteins, six from set 2 and three from set 1. The $FO_{0.5}$ values ranged from a low of 0.44 μM for BSA with α-mannosyl units (set 1) to a high of 10.6 μM for BSA with α-L-fucosyl units (set 2). Considering all nine neoglycoproteins together, the mean $FO_{0.5}$ was 5.24 μM. In only one instance (α-L-fucose) were these measurements made on neoglycoproteins containing the same sugar but attached by different chemistries. In this instance, the $FO_{0.5}$ of HD5 was 1.5 μM for the set 1 probe bearing α-L-fucose and 10.6 μM for its set 2 analog. If all six set 2 probes are taken as a group, their $FO_{0.5}$ would be 7.07 $\pm$ 1.05 μM (mean $\pm$ SEM). If the three set 1 peptides are taken as a group, their $FO_{0.5}$ would be 1.58 $\pm$ 0.68 μM (mean $\pm$ SEM). Overall, the K_d with which HD5 binds carbohydrate moieties that are covalently attached to BSA is generally between 1 and 10 μM. To put this value in perspective, high-affinity Abs typically have K_d values of 0.1–10 nM (57). This K_d corresponds to an affinity (K_a) that is 100- to 1000-fold greater, since K_a is equal to $1/K_d$. The affinity (K_d = 30–150 nM) of HD5 for the glycoproteins that we examined (gp120 of HIV-1, HSV-1 gD1, or

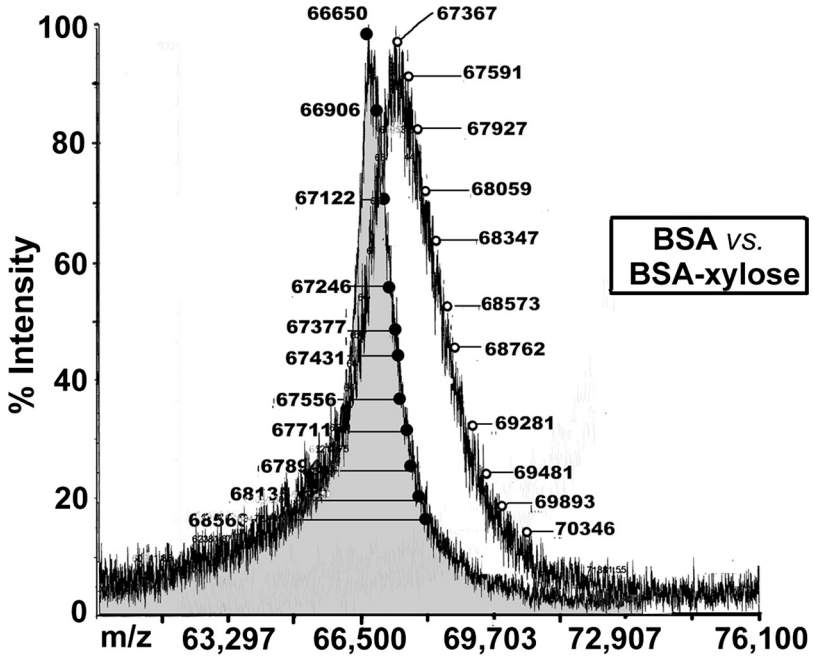

FIGURE 6. MALDI-MS analysis. The shaded *m/z* profile representing modified BSA is partially overlapped by the *m/z* profile of the set 1 xylose-bearing neoglycoprotein. The mean number of xylose residues per neoglycoprotein molecule was estimated by dividing the difference in mass between the peak values of the BSA used as carrier and xylose-bearing BSA (717 mass units) by 238 (150 for xylose + 88 for the linker group used to attach it), giving a mean value of 3.08 xylose residues per BSA.

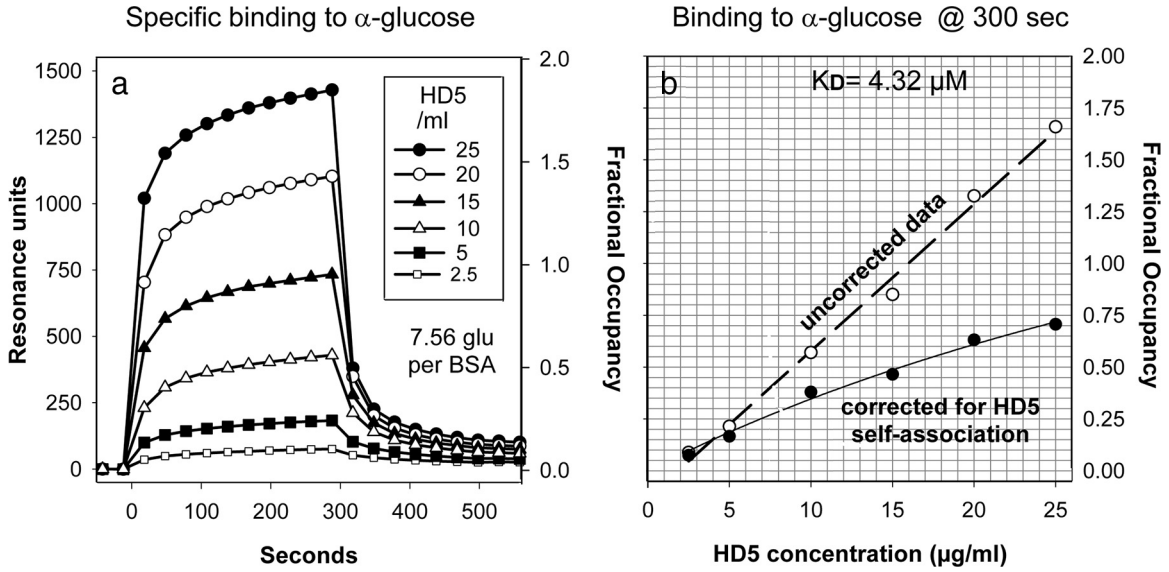

FIGURE 7. Binding of HD5 to the α-glucose residues of the neoglycoprotein. *a*, Specific binding of various concentrations of HD5 (2.5–25 μg/ml) to the α-glucose residues on 1893 RU of a group 2 neoglycoprotein containing a mean of 7.56 α-glucose residues per BSA. The control contained 1532 RU of unmodified BSA. *b* shows the fractional occupancy of glucose residues before (○) and after (●) correction for HD5 self-association. The K_d of 4.32 μM is the HD5 concentration that corresponds to a fractional occupancy of 0.5 on the corrected curve.

fetuin) falls about midway (on a $\log_{10}$ scale) between that of the aforementioned Ab-Ag interactions and its binding to simple sugars.

Binding of HD5 to sugars in solution

We used competition mode assays to evaluate the ability of HD5 to bind to carbohydrates that were not presented by a carrier but were instead free in solution. In these experiments, analytes containing 1 μg/ml of HD5 (279 nM) ± 50 mM of carbohydrate were introduced into the flow cell and the sugar's effect on the binding of HD5 to the biosensor's immobilized fetuin was monitored. Nine different sugars were tested in this way: D-fructose, D-galactose, D-galactosamine, D-glucose, D-glucosamine, D-mannose, melibiose, D-xylose, and L-xylose, with galactose and mannose representing major constituents of the glycans of fetuin. None inhibited the binding of HD5 to fetuin in the slightest degree at the concentration tested. Thus, although HD5 had considerable affinity for some of these sugars when they were attached to BSA, it showed very low affinity for the same sugars when they were in solution. This behavior is consistent with the low affinity binding of classical lectins for their cognate carbohydrates in solution (58), as exemplified by wheat germ agglutinin and *N*-acetyl-D-glucosamine

Table I. *Apparent affinity ($K_{d\ app}$) of HD5 for carbohydrate derivatives covalently linked to BSA*[a]

Sugar	Set	*n*	$FO_{0.5}$ (μM)	Residues/BSA	
α-Glucose	2	1	4.3	9.3	Chip 54
β-Glucose	2	1	8.2	9.5	Chip 54
α-Mannose	1	3	0.44	2.8	Chip 46
α-L-Fucose	2	2	10.6	10.3	Chips 51 and 52
α-L-Fucose	1	2	1.5	3.2	Chip 46
α-L-Rhamnose	2	1	3.4	10.0	Chips 51 and 52
α-Galactose	2	2	6.7	7.6	Chip 55
β-Galactose	2	1	9.2	8.8	Chip 55
α-GalNAc	1	2	2.8	3.6	Chip 46

[a] Sugars were linked to BSA by diazonium reactions in the set 1 neoglycoproteins and as *p*-isothiocyanatophenyl derivatives in those of set 2. $FO_{0.5}$ shows the concentration of HD5 that bound one-half of the sugars attached to BSA. When *n* (the number of experiments) is >1, the $FO_{0.5}$ value represents the mean.

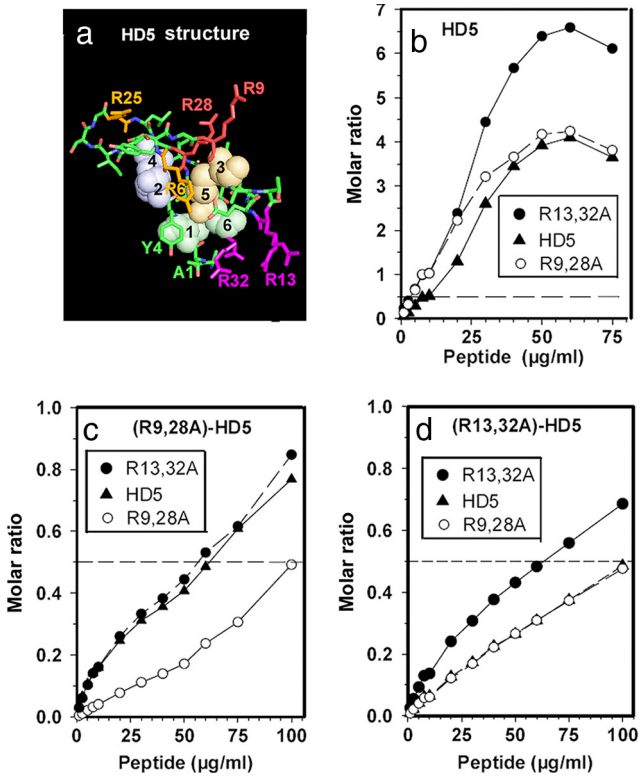

FIGURE 8. Self-association study. *a* shows the structure of HD5 and is based on a PyMol rendering of the crystal structure (Brookhaven Protein Data Bank ID: 1ZMP) of HD5 (55). The 6 cysteines of HD5 are represented by spheres, with numbers 1–6 referring to their proximity, along the backbone, to the N-terminal residue (Ala^1). The arginines are color coded as follows: Arg^9 and Arg^{28} (red), Arg^{13} and Arg^{32} (magenta), Arg^6 and Arg^{25} (orange). All other noncysteine residues are green. The biosensors contained 991 RU of immobilized HD5, 729 RU of immobilized (R13,32A)-HD5, and 1150 RU of (R9,28A)-HD5. A fourth biosensor (background) displayed the CM5 matrix. Binding is shown as molar ratio (molecules of analyte per molecule of immobilized ligand) at 300 s. Appropriate corrections were made to compensate for the slightly different masses of HD5 (3582 Da) and the R-to-A analogs (3412 Da). In panels *b–d*, immobilized ligands are boxed and solution phase analytes are identified at the *top* of the panel.

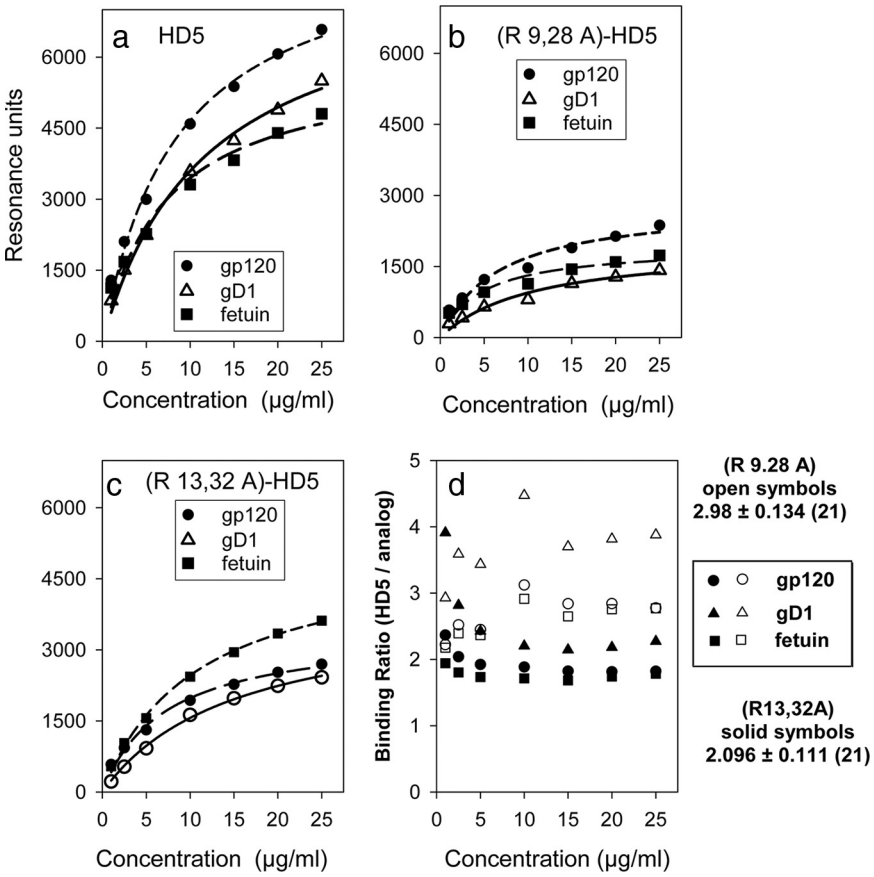

FIGURE 9. Binding of HD5 and its R9,28A and R13,32 A analogs to glycoproteins. *a*, Binding of various concentrations of HD5 to biosensors presenting 5655 RU of gp120LAV, 4296 RU of gD1, and 3911 RU of fetuin. *b* and *c*, binding of R9,28A and R13,32A analogs of HD5 to these same biosensors. *d*, ratio (HD5/analog) at each concentration. The symbols are defined within *a–c* or adjacent to *d* in each panel. No corrections for self-association or the different masses of HD5 (3582 Da) and the analogs (3412 Da) were made. The mean ± SEM ratio value was 2.096 ± 0.111 for (R13A, R32A) HD5 and 2.98 ± 0.134 for (R9A,R28A) HD5 ($n = 21$ for both). These values differed significantly ($p < 0.001$) from each other by the Wilcoxon signed rank test.

($K_a = 0.4 \times 10^{-3}$ M^{-1}), soy bean agglutinin and *N*-acetyl-D-galactosamine ($K_a = 9 \times 10^{-3}$ M^{-1}), or galectin-1 and lactose ($K_a = 5.6 \times 10^{-3}$ M^{-1}).

Arginine-deficient analogs of HD5

HD5 contains six arginine residues (Fig. 8*a*), and the side chains of arginines 13 and 32 and of arginines 9 and 28 extend from the peptide backbone, in close proximity to each other. We prepared analogs of HD5 wherein a pair of arginines, either Arg^9 and Arg^{28} or Arg^{13} and Arg^{32}, were replaced by alanine residues and tested their ability to self-associate and to bind HD5. We will refer to the former analog as (R9,28A)-HD5 and to the latter analog as (R13,32A)-HD5. Fig. 8*b* shows that at 50 μg/ml, ~6.5 molecules of solution-phase HD5, binds each molecule of immobilized HD5, even higher than the MR of 4.3 shown in Fig. 5. HD5 also bound (R13,32A) and (R9,28A)-HD5, and its subsequent self-association reached a MR of ~4.0 at 50 μg/ml.

Self-binding of the R-to-A analogs and their subsequent self-association were greatly decreased. Only at 100 μg/ml (Fig. 8*c*) did the self-binding of (R9,28A)-HD5 achieve a MR of 0.5, indicating that its affinity for itself (K_d, 29.3 μM) was 13.3-fold lower than the affinity of HD5 for itself (K_d, 2.2 μM). It required 60 μg/ml (R9,28A)-HD5 to attain a MR of 0.5 when either HD5 or (R13,32A)-HD5 were the immobilized ligands, consistent with a K_d of ~17 μM.

Fig. 8*d* shows that the binding and self-association of (R13,32A)-HD5 were also greatly impaired, relative to HD5. It required ~60 μg/ml (17.7 μM) for self-binding to reach a MR of 0.5, and 100 μg/ml (29.3 μM) for binding to HD5 or (R9,28A)-HD5 to reach a MR of 0.5. We interpret these results as indications that arginines-9,-13, −28 and −32 are intimately involved in oligomerization of HD5 at micromolar concentrations.

Fig. 9*a* documents the ability of 1–25 μg/ml of HD5 to bind the three tested glycoproteins. Fig. 9, *b* and *c*, show the same binding results for two HD5 analogs: (R13,23A)-HD5 and (R9,28A)-HD5 at defensin concentrations ranging from 1 to 25 μg/ml (~300 nM to 7.3 μM). (R13,32A)-HD5 bound each of these glycoproteins about one-half as extensively as HD5, and binding by (R9,28A)-HD5 was about one-third as extensive as binding by HD5 (Fig. 9*d*). The difference between the R-to-A analogs was statistically significant ($p < 0.001$; Wilcoxon signed rank test).

The decreased binding of the R-to-A analogs could have resulted from a reduced ability to form postbinding oligomers (Fig. 8), from a decrease in primary binding, or from a combination of both factors. To distinguish between these possibilities, we recalculated the binding data shown in Fig. 9, *a–c*, to compensate both for self-association and for the different masses of the peptides. These adjusted data appear in Fig. 10. Because the mass of the R-to-A analogs is only 4.75% below the mass of HD5, the principal adjustment involved self-association, which was minimal for the R-to-A analogs, even at 25 μg/ml, but relatively substantial for HD5 (Fig. 5). Fig. 10*a* shows that binding of gp120 by HD5 and (R13,R32)-HD5 was similar after correcting only for self-association (■) or for self-association and their mass differences (□). With both adjustments, the binding ratio, HD5/(R13,32A)-HD5, at 5–20 μg/ml was 1.068 ± 0.112 (mean ± SD). Between 5 and 20 μg/ml, (R9,28A)-HD5 bound gp120 less effectively than HD5 or its R13,32A analog, but caught up at 25 μg/ml. Thus, the main difference between the binding of (R13,32A)-HD5 and HD5 to gp120 resulted from their different propensities for self-association, whereas (R9,28A)-HD5 was also deficient in primary binding to gp120. Fig. 10, *b* and *c*, shows binding HD5 and the R-to-A analogs to glycoprotein D of HSV1 and to fetuin, respectively. The

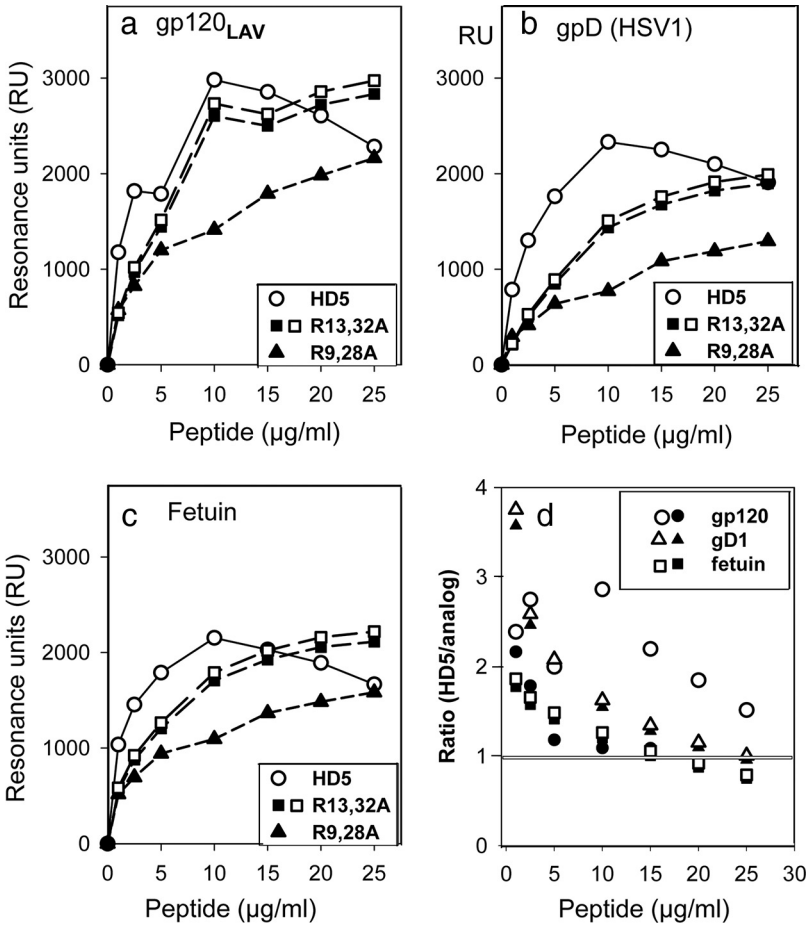

FIGURE 10. Primary binding to natural glycoproteins. The biosensors presented 5655 RU of gp120ALV, 4296 RU of glycoprotein D from HSV1, and 3911 RU of fetuin. Data in *a–c* have been corrected for the concentration-dependent self-association of each peptide, as described in the text. Data for R13,32A-HD5 are shown with (□) and without (■) additional adjustment for the mass difference between HD5 (3582) and the R-to-A analogs (3412). This adjustment involved multiplying the observed RU by 1.05 (i.e., 3582/3412). *d* shows a binding ratio (HD5:analog) at each concentration after corrections for self-association and mass differences. *d:* ○, R9,28A HD5; ●, R13,32A HD5.

findings resemble those shown in Fig. 10*a*, except that (R13,32A)-HD5 has somewhat impaired primary binding at 1–5 μg/ml. Fig. 10*d* shows the binding data from Fig. 10, *a–c*, as a ratio (HD5: analog), using data that had been corrected for self-association and mass. In Fig. 10*d*, the solid symbols represent (R13,32A)-HD5 and open symbols represent (R9,28A)-HD5. The ratios fall progressively as peptide concentrations increase, generally reaching a value of 1.0 at 15–20 μg/ml except for binding of (R9,28A)-HD5 to gp120 which only reaches a value of ~1.5 at 25 μg/ml.

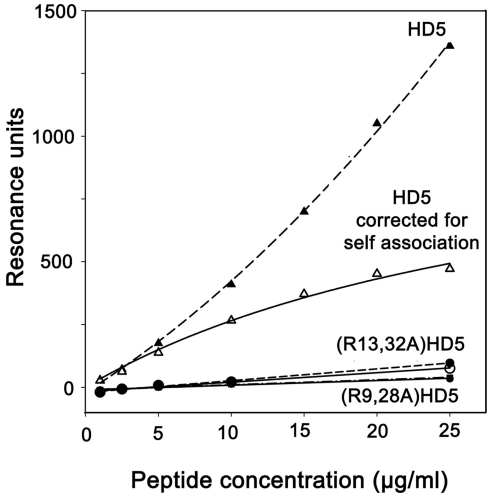

FIGURE 11. Contribution of arginine residues to carbohydrate-dependent binding of the neoglycoprotein (BSA presenting α-D-glucose moieties). Binding, shown in RU, was corrected for the binding of the peptide to carbohydrate-free BSA (solid symbols, interrupted lines) and for the effects of self-association (open symbols, solid lines). Replacement of either pair of arginines with alanines essentially abolished all glucose-dependent binding to the neoglycoprotein, as well as to unmodified BSA (not shown).

With this information in hand, we tested several neoglycoproteins to see how the R-to-A analogs would bind them. Fig. 11 shows that neither R-to-A analog showed appreciable binding to α-glucosylated BSA or to the carrier protein run as a control. Very similar results were obtained when these studies were repeated with the set 2 β-glucose-containing neoglycoprotein (data not shown). Evidently, either Arg^{9} and/or Arg^{28} and Arg^{13} and/or Arg^{32} participated in the α- and β-glucose-binding site(s) of HD5, or else their binding to nearby noncarbohydrate elements of the neoglycoprotein was essential to allow other portions of the HD5 molecule to bind the sugar moiety. We will consider these possibilities further below.

Discussion

In his 1988 essay reflecting on a century of research on carbohydrate-binding proteins, Barondes wrote: "Both classical lectins originally identified as cell agglutinins, and other carbohydrate-binding proteins that were identified by different means, may contain a second type of binding site that is specific for a noncarbohydrate ligand. This, among other findings, is changing our view of endogenous lectin functions and of the proper definition of this group of proteins." This report shows that HD5, a 3.5-kDa human α-defensin peptide, binds natural glycoproteins > BSA-based neoglycoproteins > unmodified BSA and ≫ sugars in solution. Thus, carbohydrate binding is only one facet of performance of this

multifunctional peptide. Our findings with the R-to-A analogs of HD5 (Fig. 7) strongly implicate the participation of the arginine residues of HD5 in its binding to the covalently bound sugar molecules in neoglycoproteins. The methods used to prepare these neoglycoproteins glycosylate different amino acid side chains of BSA (44). The phenylisothiocyanate derivatives used to make set 2 neoglycoproteins will primarily glycosylate the ε-amino groups of lysine, and the diazonium chemistry used to prepare set 1 neoglycoproteins will modify additional residues, especially tyrosine and histidine. A molecule of BSA contains 59 lysines, 20 tyrosines, 17 histidines, 40 aspartic acids, and 59 glutamic acids. One or more acidic amino acids are placed within 1 or 2 residues of half of the lysines (28 of 59), histidines (9 of 17) and tyrosines (10 of 20). Consequently, arginines 9, 13, 29, and 32 of HD5 may contribute to binding by interactions involving their guanidino groups and carboxyl groups adjacent to the sugar moiety attached to BSA or normally present in the naturally occurring glycoproteins. Taroni et al. (59) noted a strong propensity of certain amino acids to occur in the sugar-binding site of a lectin, especially those with aromatic rings that can pack against the hydrophobic face of the sugar, and arginine, aspartate, and glutamate residues, that can form bidentate interactions with adjacent sugar hydroxyls.

In certain respects, HD5 resembles discoidin I, a 25 kDa *Dictyostelium discoideum* lectin with a carbohydrate-binding site for *N*-acetylgalactosamine and galactose (60), plus a second binding site that binds the tripeptide, Arg-Gly-Asp (61). Protein-protein, protein-lipid, and even protein-glycopeptide interactions are also known from other lectins, involving the lectin or other sites, e.g., from Con A or human galectin-1 (62–65). Whereas discoidin-I is inherently tetrameric, HD5 instead shows concentration-dependent oligomerization, even at micromolar concentrations (Fig. 5). Acquiring multivalency via oligomerization also enables HD5 to manifest increased avidity to clustered carbohydrates. Of note in this respect, the search for natural antiviral compounds has uncovered several relatively small (8.5–12 kDa) lectins that can protect cells from infection by HIV-1 (66). Such molecules have been isolated from cyanobacteria, corals, algae, plants, or animals (66). Most of them also exist as dimers or tetramers, a sure aid to establishing di-, tetra-, or multivalent binding.

Sharon and Lis commented, as follows, on the effects of multivalency on lectin performance: "The most striking features of the lectin-monosaccharide interactions are that they are relatively weak (usually in the millimolar range) and often show relaxed specificity, when compared, for example, with that of enzyme-substrate interactions. Thus, a lectin may bind different sugars that have little in common except the orientation of a hydroxyl and an acetamide, or a few hydroxyls, for example, mannose and fucose. Nevertheless, lectins exhibit high affinity and specificity for oligosaccharides of cell surface glycoproteins and glycolipids, a prerequisite for their function as recognition molecules in biological processes. It has therefore been suggested that multiple protein-carbohydrate interactions cooperate in each recognition event to give the necessary functional affinity (or avidity) and specificity (67–71)." Sharon and Lis went on to state: "There are several possible ways, either alone or together, by which this is achieved: (a) ligand multivalency; (b) an extended binding region capable of interaction with more than just a single monosaccharide residue of an oligosaccharide,. . . and (c) clustering of several identical binding sites by formation of protein oligomers that can combine simultaneously with different, and appropriately spaced arms of a branched oligosaccharide, to separate carbohydrate chains of the same glycoprotein or to carbohydrate chains on different glycoproteins (or glycolipids) on a cell surface; (d) combination of (a) and (c)." Although these comments were not originally written about HD5, they fit our HD5 data perfectly.

Given the very large number of lectins established from at least 13 different folds (72), we will limit the remainder of this discussion to carbohydrate-binding peptides that contain fewer than 40 residues and have direct host defense properties. The smallest of these is odorranalectin (YASPKCFRYPNGVLACT), a 17-residue, L-fucose-binding peptide with one disulfide bond and a β-turn/β-sheet conformation, found in skin secretions of the frog *Odorrana grahami* (24). NMR titration suggested that Lys^5, Cys^6, Phe^7, Cys^{16}, and Thr^{17} form its fucose-binding site. Very low concentrations of odorranalectin agglutinated human RBC, bacteria, and *Candida albicans*. Its self-association has not been formally examined, but because agglutination implies multivalent binding, it must almost certainly occur.

Next in size come θ-defensins, the cyclic backbone of which contains 18 amino acid residues. These include four to six arginines plus six cysteines that form a tri-disulfide ladder cross-connecting their antiparallel β-strands (23, 73). θ-Defensins kill bacteria (15, 17), show broad-spectrum antiviral activity (5, 21, 74), and inhibit bacterial toxins, including anthrax lethal factor, a non-glycosylated zinc metalloprotease (75). Our curiosity about their carbohydrate-binding properties began after finding that retrocyclins (humanized θ-defensin peptides) protected cells from HIV-1 by preventing viral entry (74, 76, 77). Binding studies with glycoproteins involved in HIV-1 entry revealed that retrocyclins were lectins, and that carbohydrate-binding and protective efficacy against HIV-1 were highly correlated (22). Yet, their protective mechanism proved to be carbohydrate independent; resulting from binding to anionic residues in the heptad repeat domain of gp41 in a manner that prevented 6-helix bundle formation (78). Thus, like HD5, retrocyclins bind noncarbohydrate domains of glycoproteins as well as their glycans.

Small peptides with antimicrobial and lectin-like activity are found in plants (Ac-AMP2, Mj-AMP2, hevein), spider venom (SHL-1), and shrimp (penaedins). Ac-AMP2, a 30-residue peptide found in *Amaranthus caudatus* seeds (79, 80), has potent antifungal activity and binds specifically to chitin, a polymer of β-1,4-*N*-acetyl-D-glucosamine (81). Mj-AMP2 (82) is a 36-residue, cationic peptide from the seeds of *Mirabilis jalapa* and hevein is a 43-residue, multivalent, chitin-binding antifungal peptide found in the rubber tree (*Hevea brasiliensis*) (83–85). SHL-1 is a 32-residue peptide found in a spider venom (86, 87) with three disulfide bonds that form a cystine knot motif, wherein one disulfide bridge crosses between the other two. Penaedins are antimicrobial peptides found in various species of shrimp. They contain a 26-residue, chitin-binding C-terminal domain (CACYRLSVS DARNCCIKFGSCCHLVK-NH_2), the six cysteines of which form three intramolecular disulfide bonds (88, 89).

Although the placement and pairing of their cysteine residues varies, it is remarkable just how many small antimicrobial and lectin-like peptides in plants or invertebrates contain six cysteines and three intramolecular disulfide bonds. Whether these disulfides can lead to intermolecular bridges and/or redox-dependent switches between intra- and intermolecular bonding, as recently revealed for the galactoside-specific galectin CG-1B (90), is an open and interesting question. Thus, although the lectin-like properties of HD-5 may seem surprising, nature has conjured up similar surprises on many other occasions.

Disclosures

The authors have no financial conflict of interest.

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