[21] Visualization and Characterization of Receptor Clusters by Transmission Electron Microscopy

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Introduction

Multivalency is a feature common to many natural glycoconjugates, including glucosaminoglycans, mucins, and lipopolysaccharides.[1–8] Multivalent ligands often bind avidly to their target receptors. In addition, they can simultaneously bind multiple receptors and cause them to become clustered (Fig. 1).[2,9–13] Receptor clustering by multivalent ligands modulates the cell adhesion and signaling functions of many carbohydrate-binding proteins, including galectins and selectins.[14,15] Not all clusters, however, are equivalent in signaling potency or adhesion properties. Clusters that contain multiple copies of a receptor often elicit greater responses; therefore, the stoichiometry of receptor–ligand clusters can be an important determinant of activity. The relationship between stoichiometry and activity is evident in the systems that govern cellular proliferation,[16–18]

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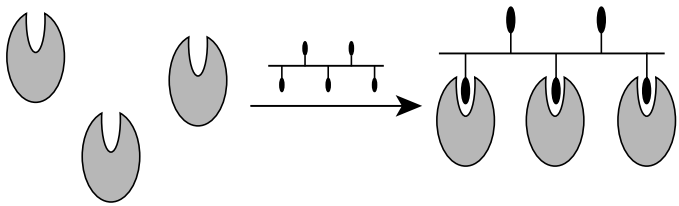

FIG. 1. Multivalent ligand-induced clustering of a receptor.

immune responses,[12,19] apoptosis,[20–23] and cell aggregation[24]: ligands that cluster more receptors are more potent. Thus, there is a need for assays that can be used to characterize multivalent ligand–receptor clusters.

Despite the importance of stoichiometry in multivalent carbohydrate–receptor interactions, methods for measuring functional valency, or the number of receptors bound to a single multivalent ligand, are not general. These methods, including immunoprecipitation,[25] spin labeling,[26] fluorescence and scanning near-field optical microscopy,[27–30] analytical ultracentrifugation, circular dichroism,[31] electrospray ionization mass spectrometry,[32,33] capillary and gel electrophoresis,[34–36] and light-scattering

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experiments,[37] are generally well-suited to the examination of high-affinity interactions between homogeneous protein populations, such as complexes formed with monoclonal antibodies. In contrast, carbohydrate receptor clustering, although a key component of signaling, is not necessarily a high-affinity event.[38] Most methods that are generally applicable to the study of carbohydrate binding tend to ignore the impact of lectin clustering and, instead, focus on the role of multivalency in enhancing functional affinity (apparent binding affinity). Affinity-based assays often fail to identify ligands with potent clustering abilities. New methods are needed for the routine analysis of glycoconjugate–receptor clusters.

Several features of carbohydrate ligand–lectin clustering must be taken into account when developing new assays. When multivalent carbohydrate binding occurs, the resultant complexes can have a wide range of functional affinities. Consequently, methods used to investigate the stoichiometries of these complexes must allow analysis of either strong or weak binding. Moreover, when the binding of a multivalent ligand results in receptor clusters that vary in stoichiometry and abundance, it is useful to have a sensitive method that can be used to visualize the entire range of clusters, including low-abundance species. Further, complexation of receptors and multivalent ligands can give rise to assemblies with high molecular weights. These large complexes often precipitate,[39,40] leading to significant constraints on solution-based approaches.

Advances in methods for single particle detection are providing new opportunities for investigating proteins.[41–45] We envisioned that applying single particle techniques to the analysis of multivalent carbohydrate–

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protein interactions would be synergistic with existing methods.[46] Specifically, we have applied transmission electron microscopy (TEM) to directly visualize individual carbohydrate ligand–lectin clusters. Like other highly sensitive methods, TEM has the advantages of nanometer resolution and a requirement for only small quantities of reagents. In addition, because individual clusters can be visualized, we anticipated that even minor species could be detected in our analyses.[46]

To explore the generality of our TEM method, we sought to investigate a series of carbohydrate receptors with diverse characteristics. Toward this goal, we chose to study the clustering of the plant lectin concanavalin A (Con A), the bacterial periplasm resident, glucose/galactose-binding protein (GGBP), and the human lectin, mannose-binding protein (MBP). In addition to their varied origins and functions, these proteins possess a range of affinities: Con A and MBP bind mannose with a K_d of approximately 1 mM,[47,48] whereas GGBP has a K_d of 0.5 μM for galactose.[49] Further, the quaternary structures of these proteins are diverse: Con A is a homotetramer, GGBP is a monomer, and MBP is a homotrimer.[50] Finally, these proteins may have important therapeutic or biotechnological potential: Con A has potent apoptotic and mitogenic activity,[51] GGBP is being explored as a potential biosensor,[52] and MBP is involved in the function of the innate immune system.[53]

We hypothesized that our TEM method would be particularly useful in exploring the effects of ligand valency on the stoichiometry of the resulting clusters (Fig. 2). We chose to focus on multivalent ligands derived from ring-opening metathesis polymerization (ROMP) because of the strong clustering activity of ligands derived from this scaffold.[38,46] To explore the influence of ligand valency on the stoichiometry of receptor clusters by TEM, we generated multivalent carbohydrate-bearing ligands with distinct valencies by ROMP (Fig. 4).[54–56] We generated ROMP-derived multivalent ligands displaying a defined number of mannose or galactose

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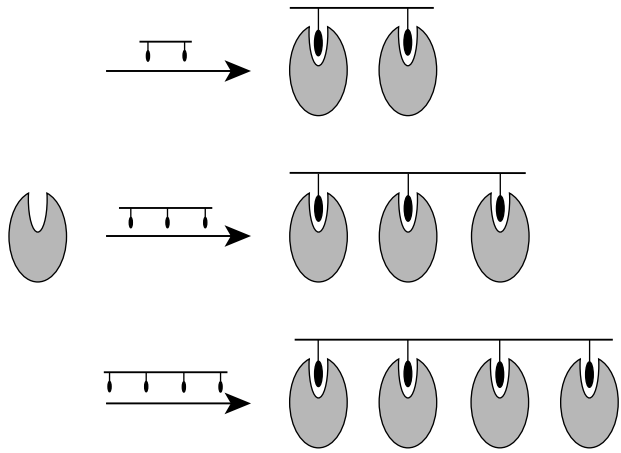

FIG. 2. Illustration of the ligand valency dependence of receptor cluster sizes. Increases in the valency of the multivalent ligand increase the maximum stoichiometry of the receptor and ligand in the resulting complex.

derivatives. Mannose is a ligand for Con A and MBP and galactose binds to GGBP. We envisioned that, if the ligands promote lectin clustering, increasing the valency of the synthetic ligands would increase the number of copies of lectin incorporated into the resulting clusters.[46]

Transmission Electron Microscopy Protocol

General Considerations

The resolution of TEM is greatly increased by using particles with high electron density, such as heavy metals. Therefore, we label biotinylated receptors with an electron-dense streptavidin–gold nanoparticle by taking advantage of the tight interaction between biotin and streptavidin (Fig. 3). Here, we describe the biotinylation of Con A, GGBP, and MBP and present a protocol for the subsequent TEM-based investigation of multivalent ligand interactions with these biotinylated receptors. The sources of the receptors are as follows: Con A is obtained from Vector Laboratories (Burlingame, CA), GGBP is produced in *Escherichis coli* by the osmotic shockate method as previously described,[57] and recombinant MBP is produced in *E. coli* and purified according to previously described procedures.[58]

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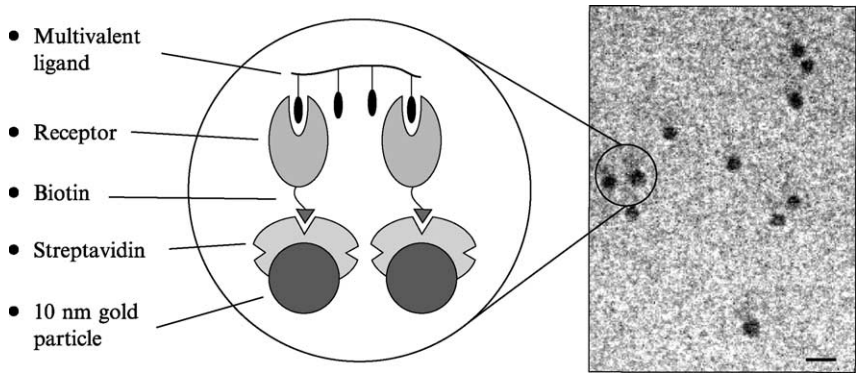

FIG. 3. Experimental design of TEM-based visualization of single receptor clusters. Minimally biotinylated receptor is bound to a multivalent ligand via its carbohydrate-binding site and a gold nanoparticle via its biotin group. At the right is a sample field from an experiment in which biotinylated Con A was treated with steptavidin–gold conjugates and a multivalent ligand. A cluster of two is shown in the circle. Bar: 20 nm.

Biotinylation Reactions

To minimize nonspecific effects, the receptor of interest should be modified with a low level of biotin (ideally one copy of biotin per receptor). Commercial reagents are available for biotinylating receptors and the subsequent determination of the extent of biotinylation. Here we provide a brief synopsis of these protocols for the biotinylation of the target receptors (Con A, GGBP, and MBP).

A solution of receptor (1 mg ml^{-1}) in 0.1 *M* sodium borate at pH 8.8 is biotinylated for 12 h at room temperature, using final biotinylating reagent concentrations ranging from 0 to 500 $\mu g\ ml^{-1}$ in a 1-ml final volume. The biotinylating reagent is sulfosuccinimidyl-6-(biotinamido)hexanoate (EZ-Link sulfo-NHS-LC-biotin; Pierce, Rockford, IL). The biotinylation reactions are quenched with a 1 *M* aqueous solution of NH_4Cl. Excess biotinylating reagent is removed by extensive dialysis against 10 m*M* HEPES, pH 7.0, at 4°. The concentration of receptor after dialysis is determined by the Bradford assay, using bovine serum albumin (BSA) as a standard. Molar ratios of biotin to receptor are determined with 2-(4′-hydroxyazobenzene)benzoic acid (HABA; Pierce) according to the manufacturer's specifications.

A Con A–biotin conjugate with a biotin-to-Con A tetramer ratio of 2:1 is derived from a reaction with a starting biotinylating reagent concentration of 5 $\mu g\ ml^{-1}$. Modified GGBP and MBP are labeled with a protein-to-biotin ratio of 1:1. These results are obtained with a biotinylating

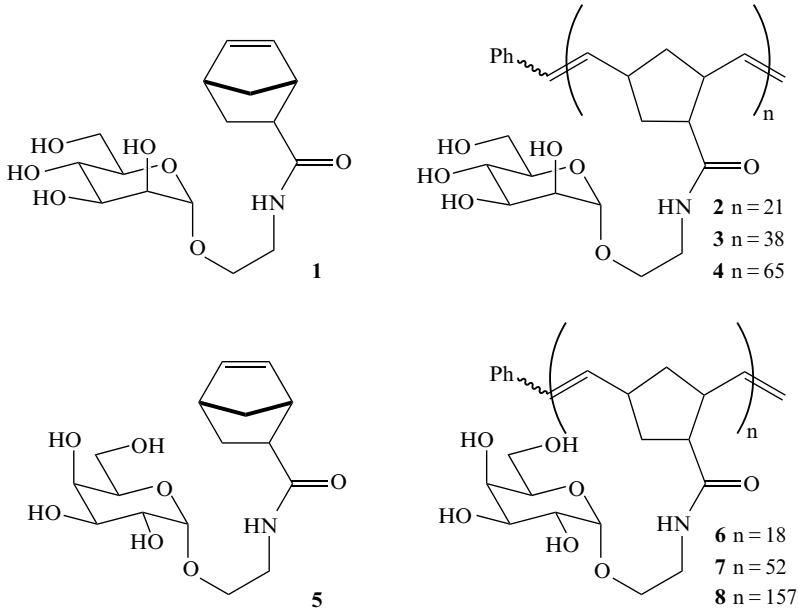

FIG. 4. Chemical structures of mannose- and galactose-bearing synthetic glycoconjugates. Monomers **1** and **5** were used to generate **2**–**4** and **6**–**8**, as previously described.[56] The average valency (degree of polymerization) of the polymers (*n*) is shown.

reagent concentration of 25 and 100 μg ml^{-1}. The conjugates are stored at -20° and are used for TEM experiments.

Electron Microscopy

Complexes between ligand and biotinylated receptor are assembled in solution and then visualized by placing them on grids for electron microscopy. Ligand (0.75 μM saccharide) is added to biotinylated receptor (Con A, 2.3 μM; GGBP, 1.8 μM; MBP, 1.9 μM) in 5 μl of phosphate-buffered saline (PBS), pH 7.2. Ligand concentrations are based on the total saccharide residue concentration, not on polymer concentration. Complexes between ligand and receptor are allowed to form for 15 min at room temperature before streptavidin–10 nm gold (3.0 μM) is added. This mixture is transferred to Formvar-treated copper grids. After an additional incubation of 10 min, excess liquid is removed. TEM is performed on a LEO (Oberkochen, Germany) Omega 912 energy-filtering electron microscope (EFTEM) outfitted with a ProScan slow scan charge-coupled device (CCD) camera. A convenient number of particles per field is obtained at a magnification of $\times$12,500.

Stoichiometry Optimization

Monomers **1** and **5** (Fig. 4) serve as important controls for optimizing the experimental conditions. These monovalent ligands are incapable of nucleating clusters; therefore, these compounds can be used to determine conditions for optimal particle distribution. High concentrations of gold particles should be avoided, as it can be difficult to discern coincidental adjacent placement from multivalent ligand-induced clustering. In addition, low concentrations should be avoided, as this prevents collection of statistically meaningful data. Subsequently, a short series of dilution experiments are performed in the presence of monomeric ligands **1** and **5** to optimize the reagent concentrations. Under optimal conditions, a molar ratio of 1:1.3 (receptor:gold particles) is used. A molar ratio of 1:3 (ligand:receptor) is used for Con A and a molar ratio of 1:2.5 is used for GGBP and MBP. These conditions yield approximately 10–100 particles per field.

Image Analysis

Complex formation is determined manually, using the measuring tools in Adobe Photoshop 5.0. The stoichiometry of individual clusters is scored by determining the number of gold particles that can be simultaneously contacted by a 25-nm line. Thus, clusters of two are defined as two gold particles within 25 nm and clusters of three, four, and five proteins are defined as three, four, or five gold particles within this distance, respectively. This distance (25 nm) is determined on the basis of the maximum distance between the terminal saccharides on a fully extended polymer generated by ROMP, as previously described.[46,56] Although the measurement distance used is specific for a polymer of intermediate size, the results of the manual counting experiments are not significantly altered when the distance is doubled (data not shown). This independence is likely due to the gold particles being well-distributed in the observation fields.

Three experiments are performed on separate days, using freshly prepared grids. On each day, 80–150 gold particles are counted from approximately 10–20 images of random fields for each treatment. The percentage of each complex type (unclustered, cluster of two, cluster of three, or cluster of four) is determined on each day. The final reported percentages are averages of the three experiments (Fig. 5). The 10-nm gold particles are used as a convenient internal size standard. On occasion, large aggregates of gold are observed in the preparations; these particles are discounted from the analysis and can be removed by centrifugation before sample preparation.

Discussion

Analysis of the TEM images revealed that increasing the length of the multivalent ligand increases the number of copies of each of the bound lectins (Fig. 5). For example, three copies of Con A could assemble on multivalent ligand **4**, whereas the shorter oligomer **2** typically complexed only two receptors. Previous experiments have indicated that high-valency ligands have a greater avidity for Con A.[56] These TEM results suggest that enhanced avidity could be derived from the ability of these materials to assemble larger clusters of Con A. In addition, these results provide a means to quantitate the stoichiometry of these clusters and provide visual images of the complexes proposed to mediate high-avidity binding.

As expected, each receptor–ligand pair produced a distribution of possible stoichiometries. For example, addition of the 157-mer galactose ligand **8** to GGBP yielded an increase in the percentage of clusters of two (12% of the total particles), but also a significant number of clusters of three (2.4%). Long polymers were also required to alter the proportions of clustered MBP; the longest mannose polymer used (**4**) resulted in an increase in the proportion of clusters of two and three. In addition, compounds **2** and **4** were able to cluster three and four copies of MBP; no clusters of these sizes were observed in the absence of the multivalent ligands. The presence of these low-abundance clusters (less than 5–10%) would be difficult to detect in other assays, but individual clusters are discernible by TEM. This is a significant advantage, because subsets of the population could make disproportionately high contributions to the biological activity of the sample. Therefore, resolving populations may be important for understanding the consequences of receptor clustering.

We had previously attempted to explore the clustering of Con A by light-scattering experiments; however, these experiments were unsuccessful because of the insolubility of the resulting clusters. Our TEM method, in contrast, provides a simple means to investigate and visualize ligand-mediated Con A clustering. The low protein concentrations required for these experiments may favor soluble clusters. In addition, because receptors are viewed on surfaces and not in solution, we do not anticipate that solubility is required.

In the application of the TEM method described here, information about the approximate size of the ligand was used to analyze the data. In less structurally characterized systems, access to this information may be limited. However, for chemically defined glycoconjugates, which are used in many applications, structural approximations are often available. Moreover, if some aspect of the ligand is systematically varied, useful information about its ability to promote clustering can be obtained in the absence of structural approximations.

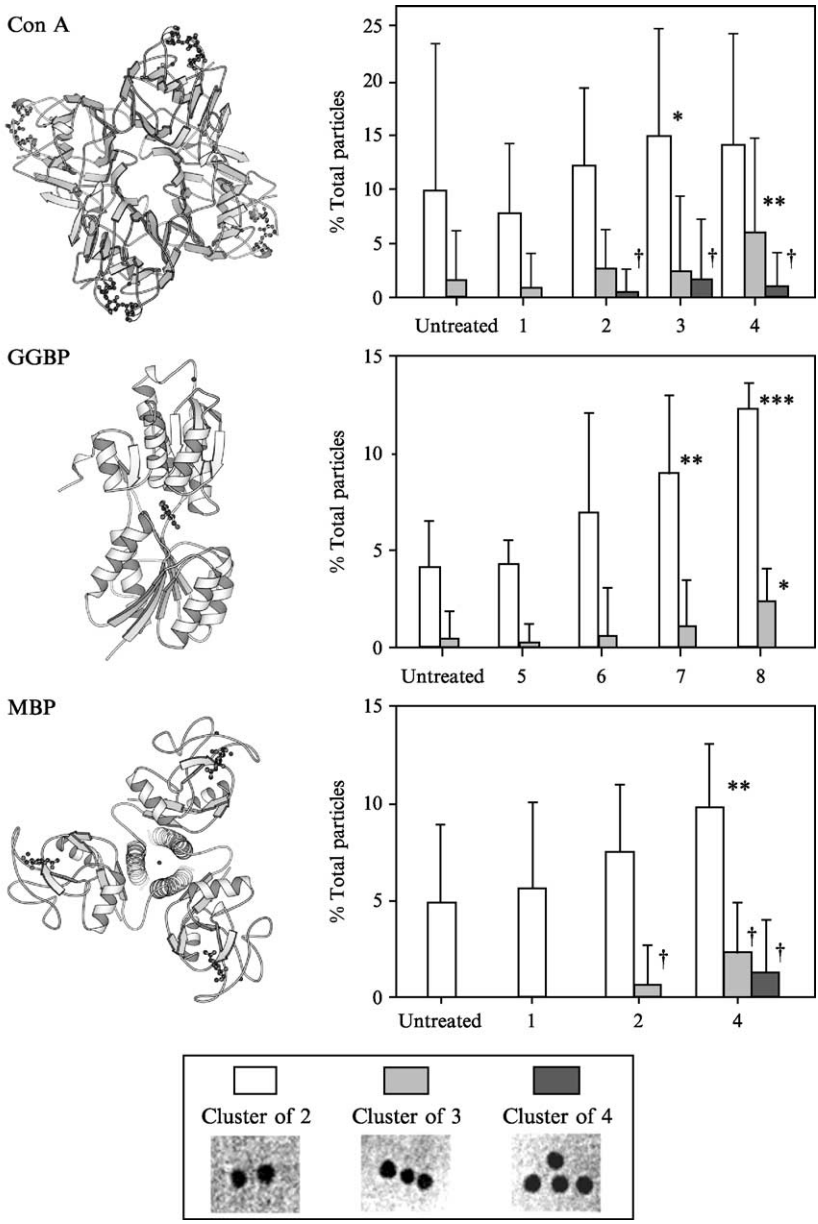

FIG. 5. Ligand valency determines the number of receptors bound to a ligand. The protein structures of receptors Con A, GGBP, and MBP are shown next to the data relevant to their clustering by multivalent ligands. The graphic legend displays possible cluster sizes from clusters of two to four and the images are representative clusters of that type. The data (SD) are presented as a percentage of the total number of particles counted. For populations with

Conclusions

The prevalence of multivalency in protein–carbohydrate interactions mandates that new methods to characterize multivalent binding be developed. Although many assays focus on assessing the apparent affinity of a multivalent carbohydrate–lectin interaction, many multivalent ligands promote lectin clustering. Because the clustering of a lectin can interfere with or augment its function,[15,24] methods to characterize lectin–ligand assemblies are needed. The TEM method described here facilitates the visualization and characterization of such clusters. In addition, using this technique, the effects of ligand valency on cluster size can be investigated. We envision that future applications may focus on the effects of other aspects of ligand architecture on receptor clustering, such as binding epitope density[40] or shape of the multivalent display.[38] We anticipate that our approach is general and that diverse synthetic glycoconjugates and natural multivalent ligands can be subjected to TEM analysis.

The results of TEM experiments should be interpreted as providing a lower bound to the stoichiometry of individual clusters. As performed here, we have optimized conditions to visualize individual clusters; to be useful this must be performed at dilute conditions so that second-order clustering is not observed and individual clusters are resolved. Therefore, these results may not represent total populations of bound versus free ligand. Instead, they represent the number of receptors that can bind to individual multivalent ligands. This is distinct from methods, such as quantitative precipitation, that yield only the final stoichiometry of precipitated proteins that may be incorporated into an extended lattice.[59]

Another potential use of this TEM method is in exploring heterogeneous multireceptor clusters. Gold nanoparticles of various sizes are commercially available; therefore, each component of a proposed multiprotein assembly could be individually labeled. Subsequent reconstitution of the labeled proteins could allow visualization of the stoichiometry of the heterogeneous complex. Results from such experiments can provide insight into the function of multireceptor clusters.

overlapping error, p values were calculated and are noted (Con A: $^{*}p < 0.3$; $^{**}p < 0.2$; $^{***}p < 0.1$. GGBP and MBP: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$). Species that are not found in untreated experiments are noted (†). The PDB codes for the protein structures shown are as follows: Con A is 1CVN,[60] GGBP is 1GLG,[61] and MBP is 1BCH.[62]

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[22] Exploring Kinetics and Mechanism of Protein–Sugar Recognition by Surface Plasmon Resonance

By MILI KAPOOR, CELESTINE J. THOMAS, KIRAN BACHHAWAT-SIKDER, SHILPI SHARMA, and AVADHESHA SUROLIA

Introduction

The study of biomolecular recognition is of basic importance in understanding processes of molecular recognition and biological function. Lectins are a class of nonenzymatic carbohydrate-binding proteins found ubiquitously in nature. These lectin–ligand attachments are critical in several biological processes such as cell signaling, life cycling of pathogens, fertilization, and inflammatory responses and are put to use in biomedical research as carbohydrate probes based on the binding to surface sugars.[1,2] The kinds of forces involved in lectin–sugar interactions are generally weak and include noncovalent forces, yet the specificity required for a given cellular adhesive event is great.

Determination of kinetic/thermodynamic parameters involved in protein–sugar interactions at the molecular level provides a basic framework for understanding the mechanism of recognition.[3–6] Conventional analysis often relies on techniques requiring large amounts of proteins and/or ligands or both and often entails modification to incorporate a

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