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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
COMMUNICATION
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Toward Bioinspired Galectin Mimetics: Identification of Ligand-Contacting Peptides by Proteolytic-Excision Mass Spectrometry

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S *Supporting Information*

ABSTRACT: Clinically relevant bioactivities of human galectins (adhesion/growth-regulatory galactoside-specific lectins) inspired the design of peptides as new tools to elicit favorable effects (e.g., in growth control) or block harmful binding (e.g., in tissue invasion). To obtain the bioinspired lead compounds, we combined a proteolytic fragmentation approach without/with ligand contact (excision) with mass spectrometric identification of affinity-bound protein fragments, using galectin-1 and -3 as models. Two peptides from the carbohydrate recognition domains were obtained in each case in experimental series rigorously controlled for specificity, and the [157−162] peptide of galectin-3 proved to be active in blocking lectin binding to a neoglycoprotein and to tumor cell surfaces. This approach affords peptide sequences for structural optimization and intrafamily/phylogenetic galectin comparison at the binding-site level with a minimal requirement of protein quantity, and it is even amenable to mixtures.

The increasing insight into the broad functionality of protein-(lectin)−carbohydrate recognition has engendered a growing potential for medical applications.[1] Because of their strategic positioning at branch ends and dynamic physiological remodeling underlying key decisions on the cellular fate, galactosides are major contact points for endogenous lectins.[2] Beyond becoming promising candidates as functional glycobiomarkers with predictive power, the role of galactosides as bioactive ligands defines a route toward new lead compounds for glycan-directed drug design derived from lectins.[3] In the family of human galectins, the prominent member galectin-1 is known as a potent effector, depending on cell type and counter-receptor presence; for example it exerts cell cycle control and induces anoikis or acts as a negative prognostic factor by enhancing tissue invasion.[4] Evidently, the availability of peptides mimicking the target specificity of the galectin would enable tests for clinical applicability, either to elicit antitumor signaling or interfere with promalignant processes.

The example of the α/θ-defensins with a minimal size of 18 amino acids, along with plant mini-lectins such as hevein, attests to the biological potential of peptides toward this end.[5] It is underscored by progress with custom-made design in this direction. Through exploitation of well-defined binding motifs for heparan sulfate/hyaluronic acid, efficient peptides were prepared against these polyanionic glycan targets.[6] Likewise, biopanning of engineered phage populations led to carbohydrate-binding peptides, also against neutral glycan epitopes from glycoproteins and glycolipids, although their affinity when free in solution did not reach a high level.[7] The bioinspired design of peptides, which constitute major parts of the contact site of a receptor (antibody, lectin), is becoming a viable route, as has been shown for an antibody specific to ganglioside GD2 and three siglecs.[8] Relevant peptide sequences could also be derived from Asp-N endoproteinase digests of leguminous lectins, where a Ca^{2+} ion is crucial for structural organization of the contact site for the sugar.[9] In the case of galectins, however, the minimal folding unit for its activity (i.e., galectin-3) was defined by phage display to represent the carbohydrate recognition domain of 136 amino acids.[10] This observation would preclude any bioinspired design of galectin-mimicking peptides. In order to address this problem, we sought to develop a general method for identifying peptides with activity toward the β-galactoside core. We report here a rigorously controlled approach for identifying carbohydrate-binding peptides from a lactose-bearing affinity matrix by a combination of proteolytic excision and mass spectrometry. Previously, mass spectrometric and proteolytic approaches have been successfully employed for the identification of peptide-epitope and paratope structures from immobilized antibody−antigen complexes,[11] thus encouraging experiments with the β-sandwich-fold galectins.

In the first series of experiments, 50 μg of human galectin-1[12] was bound to 200 μL of affinity matrix (lactosylated Sepharose 4B) in phosphate-buffered saline, and any unbound material was removed by thorough washing. Extensive in situ proteolytic digestion of bound galectin-1 was performed (3 h at 37 °C) with a trypsin/galectin ratio of 1:100, followed by complete removal of all unbound tryptic peptides (see Figure S1 and Table S1 in the Supporting Information). A total of 30 mL of buffer was used to ensure that even weakly bound material would be removed prior to competitive elution with buffer solution containing 0.3 M lactose. MALDI-MS analysis of the elution fraction yielded two distinct tryptic peptides (Figure 1 top). Thus, the elution with cognate

Received: March 3, 2011
Published: August 23, 2011

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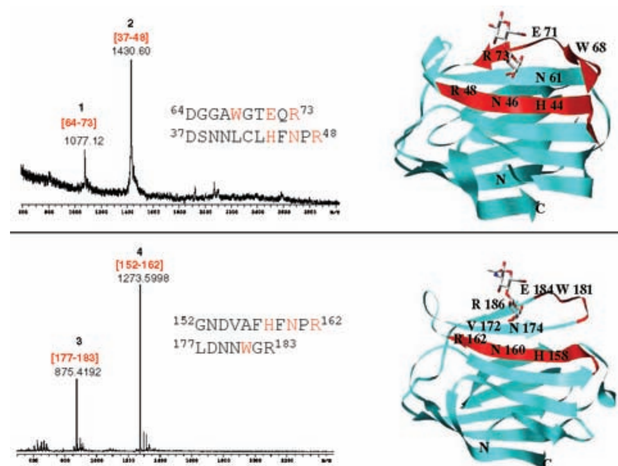

Figure 1. Proteolytic excision for complexes of lactose with (top) galectin-1 and (bottom) galectin-3. (left) MALDI-MS of elution fractions with signals of identified peptides. (right) X-ray crystal structures (PDB entries 1W6O and 1A3K), showing the identified peptides in red and the amino acids that are in direct contact with the carbohydrate in bold.

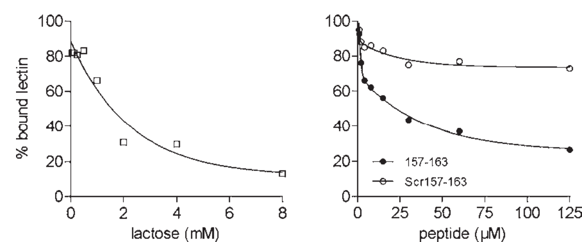

Figure 2. Inhibition of binding of human galectin-3 (15 μg/mL) to surface-immobilized neoglycoprotein (lactosylated bovine serum albumin) (250 ng/well) by (left) lactose and (right) the two listed peptides under experimental conditions described in detail previously.[16]

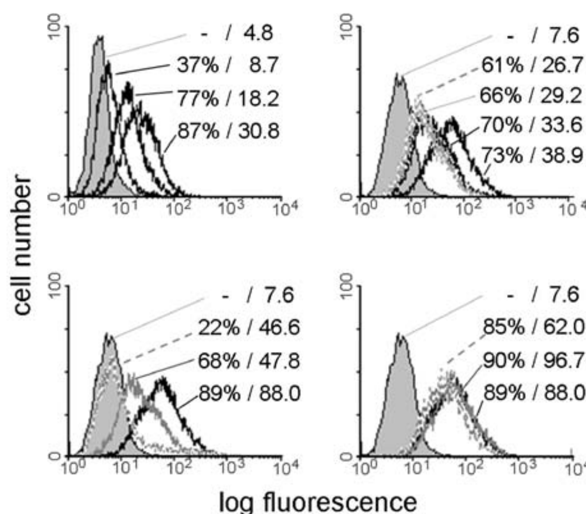

Figure 3. Semilogarithmic representation of fluorescent surface staining of cells of the human colon adenocarcinoma line SW480 with labeled human galectin-3. The concentration dependence for the probe (galectin-3 tested at 5, 10, and 20 μg/mL; left) and carbohydrate dependence of binding (lactose as an inhibitor tested at 0.5, 1, 2, and 10 mM using a constant galectin-3 concentration of 20 μg/mL; right) are shown in the top panel. Peptide 157−163 (bottom panel, left) and a scrambled peptide 157−163 (bottom panel, right) were tested at 20 μg/mL galectin-3. Their concentrations were 0.5 mM (gray line) and 1 mM (dotted line) or 0.5 mM (gray line) and 2 mM (dotted line), respectively. Quantitative data on the percentage of positive cells and mean channel fluorescence are given in each panel. The shaded areas represent the control values in the absence of lectin and the black lines the values in the absence of inhibitor (100% values). Data for the two tested peptide concentrations are listed. Experimental conditions have been given in detail previously.[17]

sugar displaced bound peptides from the immobilized ligand, raising expectations that they are parts of the contact site. Indeed, the two peptides 1 and 2 covered positions 64−73 (a sequence stretch with the central Trp residue for C−H/π interactions with galactose[12]) and positions 37−48, respectively (Figure 1 top). Notably, the relative abundances of peptide ions in MALDI-MS do not reflect their relative composition in quantitative terms;[13] the higher abundance of peptide 2 (37−48) may be explained by its comparatively high basicity. Together, these two peptides harbor the key amino acids in contact with the ligand,[12] thus representing bioactive sequence parts of galectin-1, in agreement with previous results by mutational analysis of galectin-1.[14] To support this conclusion, binding experiments on lactose were performed with peptides 1 and 2 obtained by solid-phase peptide synthesis. In accordance with their activity after in situ digestion of the lectin, both synthetic peptides bound to the affinity resin and were detected by MALDI-MS after elution with lactose (Figure S3).

To ascertain this approach further, an identical set of experiments was performed with the carbohydrate recognition domain of human adhesion/growth-regulatory galectin-3, for which a complete view of the dynamics of protein−carbohydrate recognition has recently been accomplished.[15] Again, two specific peptides, 3 [Gal-3(152−162)] and 4 [Gal-3(177−183)], out of the complete set of tryptic peptides (Table S2) were recovered and identified by MALDI-MS of the elution fraction (Figure 1 bottom). Notably, the same two peptides were also identified when trypsin digestion was performed with galectin-3 in solution and the resulting mixture of tryptic peptides was applied to the affinity matrix (Figure S3). As for galectin-1, the isolated peptides cover most of the amino acids involved in hydrogen bonds and van der Waals interactions with the cognate carbohydrate, as revealed by knowledge-based dynamic modeling (Figure S4). On the basis of this information, peptide 3 (152−162) could even be trimmed to a heptapeptide (157−163) to pinpoint its minimal size. For peptide 3, the presence of Trp is essential, and truncation to a pentapeptide impaired its affinity (Table 3), as was also documented in quantitative terms by using a surface acoustic wave (SAW) biosensor[11i] that was similarly applied for the galectin-1-derived peptides (Table S4).

In addition to ascertaining the interaction with the the affinity resin, we set out to inspect the behavior of peptide 157−163 in binding assays of increasing biorelevance, in competition with labeled full-length galectin-3. When tested in a solid-phase system, the peptide 157−163 was able to reduce galectin-3 binding to lactose presented by a surface-adsorbed neoglycoprotein (Figure 2). Of even higher relevance, this peptide specifically interfered with the binding of galectin-3 to cell surfaces (Figure 3). In addition, a series of negative control experiments was performed for both galectin-1 and -3 with all of the synthetic peptides and lactose-free Sepharose (see the example in Figure S6) as well as with

immobilized sucrose/maltose, and their complete lack of affinity was ascertained, as also observed for peptides with sequence alterations (Figure S7).

The results shown here have revealed that bioactive peptides could be identified after proteolytic cleavage of two members of the human galectin family upon binding to immobilized lactose. The identified peptides consist of two sequence stretches that provide the main interactions between the hololectin and the ligand. Further work presently under way in our laboratory using other galectins has fully confirmed the validity of the proteolytic-excision mass spectrometry approach. This experimental evidence provides the basis for ensuing work on bioactive peptides from galectins and gives direction to optimization of the affinity and selectivity (e.g., by introducing suited non-natural amino acids and generating clustered presentations), which are also pivotal to elicit biosignaling.[18] Moreover, this approach, which requires only minimal protein quantities, presents promising application perspectives for the mapping of (i) extended binding sites (e.g., the contact area for ligands larger than lactose, such as the pentasaccharide of ganglioside GM1 in neuroblastoma growth regulation and T cell communication[4b,19]) and (ii) intrafamily/phylogenetic divergence (e.g. for the different domains in tandem-repeat-type galectins).

■ ASSOCIATED CONTENT

Supporting Information. Identification of ligand-binding peptides from galectin-1 and -3; synthesis of galectin-derived and control peptides; affinity-MS characterization and quantitative K_D measurements of synthetic galectin-derived peptides; mass spectrometry and tryptic peptides; modeling of peptide−lactose contacts in galectin-3; controls; Tables S1−S4; Figures S1−S7; and complete ref 4d. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We are grateful for generous funding from the EC for the GlycoHIT Consortium, the Deutsche Forschungsgemeinschaft, and the German Academic Exchange Service (DAAD).

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