Galectin-3 Precipitates as a Pentamer with Synthetic Multivalent Carbohydrates and Forms Heterogeneous Cross-linked Complexes

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Galectin-3 is unique among the galectin family of animal lectins in its biological activities and structure. Most members of the galectin family including galectin-1 possess apoptotic activities, whereas galectin-3 possesses anti-apoptotic activity. Galectin-3 is also the only chimera type galectin and consists of a nonlectin N-terminal domain and a C-terminal carbohydrate-binding domain. Recent sedimentation equilibrium and velocity studies show that murine galectin-3 is a monomer in the absence and presence of LacNAc, a monovalent sugar. However, quantitative precipitation studies in the present report indicate that galectin-3 precipitates as a pentamer with a series of divalent pentasaccharides with terminal LacNAc residues. Furthermore, the kinetics of precipitation are fast, on the order of seconds. This indicates that although the majority of galectin-3 in solution is a monomer, a rapid equilibrium exists between the monomer and a small percentage of pentamer. The latter, in turn, precipitates with the divalent oligosaccharides, resulting in rapid conversion of monomer to pentamer by mass action equilibria. Mixed quantitative precipitation experiments and electron microscopy suggest that galectin-3 forms heterogeneous, disorganized cross-linking complexes with the multivalent carbohydrates. This contrasts with galectin-1 and many plant lectins that form homogeneous, organized cross-linked complexes. The results are discussed in terms of the biological properties of galectin-3.

Galectin-3 is a widely studied member of the galectin family of β-galactoside-specific animal lectins (1–3). It is the only chimera type galectin and possesses a nonlectin N-terminal domain linked to a C-terminal carbohydrate recognition domain (CRD)‡ (2, 4). The amino acid sequence of galectin-3 varies from 243 to 286 amino acids depending on the number of tandem repeats of a peptide sequence rich in proline, glycine, and tyrosine residues in the N-terminal domain, which is species-dependent (5). In the mammalian protein, there is also a conserved 18-amino acid N-terminal peptide sequence preceding the proline/glycine/tyrosine-rich repetitive domain that appears to have its own functions (5). The C-terminal CRD domain of galectin-3 is homologous to that of galectin-1 and other members of the galectin family (5, 6). Although the x-ray crystal structure of intact galectin-3 has not been determined, the x-ray crystal structure of the C-terminal CRD domain of galectin-3 has been solved (7) and shown to be a monomer and not a dimer as observed for the x-ray crystal structures of galectins-1 (8), -2 (9), and -7 (10).

Galectin-3 appears to possess many biological activities. It has been implicated in the regulation of the cell growth (11), leukocyte activation (13-16), induction of endothelial cell morphogenesis and angiogenesis (17), cell adhesion (18, 19), and pre-mRNA splicing (20). Galectin-3 is also reported to be involved in colon cancer metastasis (21) and brain tumor progression (22). Galectin-3 shows significant homology with the Bcl-2 class of proteins, and like Bcl-2, galectin-3 possesses anti-apoptotic activity in a variety of cells (23) in contrast to the apoptotic activities of many other members of the galectin family including galectin-1 (24). Because many of the biological properties of galectin-3 depend on its carbohydrate recognition properties (5), it is important to understand its mechanism(s) of binding to cellular carbohydrates.

The x-ray crystal structure of the CRD of galectin-3 has been reported (7) but not the structure of the intact molecule. The CRD alone binds lactose but lacks the hemagglutination activity and cooperative binding associated with intact galectin-3 (25). This suggest that the N-terminal domain is important for aggregation of the protein. Indeed purified N-terminal fragments have been found to self-associate (26). Evidence that galectin-3 self-associates in the absence of saccharides was reported using chemical cross-linking experiments (25, 27) and gel electrophoresis (28). The structures appeared to be dimers when examined by electron microscopy (28). In addition, the hemagglutination activity (29) and cooperative binding of galectin-3 to immobilized IgE (25) and laminin (30) also suggest that the lectin exists as an oligomer in the presence of a multivalent glycoconjugate. However, gel filtration studies failed to detect an oligomeric form of the lectin (25, 30). More recently, the quaternary structure of recombinant murine galectin-3 in solution was directly determined by sedimentation velocity and equilibrium measurements, and the results show that the lectin is predominantly a monomer (31). Hence, to date there is no clear understanding of the relationship between the quaternary structure of galectin-3 and its multivalent carbohydrate binding properties.
In the present study, quantitative precipitation, electron microscopy, and kinetic data have been obtained for binding and precipitation of murine recombinant galectin-3 with a series of divalent carbohydrates possessing terminal LacNAc residues. Quantitative precipitation studies indicated that the lectin precipitates as a pentamer in the presence of the divalent carbohydrates in Fig. 1 and that the kinetics of precipitation are fast. These observations suggest that a small amount of protein oligomer is in rapid equilibrium with the predominant monomer in solution and that mass action equilibria quickly convert the monomer to pentamer, which precipitates with the carbohydrates. Mixed quantitative precipitation experiments indicated that galectin-3 forms heterogeneous cross-linked complexes with the carbohydrates, and electron microscopy suggests that these complexes are structurally disorganized. These findings provide important new insights into the structure-activity properties of galectin-3.

EXPERIMENTAL PROCEDURES

Materials—Fetuin and lactose were obtained from Sigma. Asialofetuin was prepared from fetuin by desialylation and purified as described earlier (32). Asialofetuin-Sepharose was prepared by following a published procedure (33). Synthesis of the pentasaccharides in Fig. 1 have been reported (34). The structures and purity of carbohydrates were established by 1H NMR spectroscopy. All other reagents were of analytical grade.

Galectin Preparations—The plasmid pcCBP35s with cDNA for murine galectin-3 was a gift from John L. Wang (East Lansing), and the protein was expressed as described in the literature (35). The protein showed a single band by SDS-PAGE. Galectin-3 batches were found to have different $\varepsilon$ values by isothermal titration microcalorimetry using LacNAc as a ligand. Therefore, each batch of galectin-3 was subjected to isothermal titration microcalorimetry and electrospray ionization matrix-assisted laser desorption ionization and negative ion mode using a Shimadzu UV-1601 spectrophotometer. A buffered solution of the protein in a cuvette was rapidly mixed with a stock solution of a given saccharide in the same buffer at room temperature, and the light scattering was measured at 420 nm.

Electron Microscopy—Negative stain electron microscopy was performed by placing the appropriate amount of the precipitate on 300-mesh carbon-coated Parlodion grids that had been freshly glow discharged for 2 min. The samples were touched to filter paper, floated on a drop of 1% phosphotungstic acid, pH 7.0, and blotted immediately. The samples were observed at 80 kV in a JEOL 1200 EX electron microscope.

RESULTS

Precipitation of Galectin-3 with Asialofetuin, Fetuin, and Multivalent Carbohydrates—Fig. 2A shows the rapid onset of light scattering at 420 nm upon mixing 20 $\mu$M galectin-3 with 7 $\mu$M ASF, a glycoprotein of molecular mass 48,400 Da (39) containing three N-linked triantennary complex carbohydrate chains with terminal LacNAc residues (40) and three O-linked disaccharide chains with terminal Gal residues (41). The presence of 10 mM LacNAc inhibited the light scattering. Precipitation of ASF was also observed at 10 $\mu$M galectin-3 (not shown). These results demonstrate that galectin-3 cross-links and precipitates with ASF.

Similar results were obtained with fetuin (Fig. 2B), although the time course of precipitation was slightly slower than that for ASF. Fetuin differs from ASF in having a mixture of 2,3- and 2,6-sialylation on the terminal LacNAc residues (40). The slower kinetics of precipitation of fetuin by galectin-3 may be due to reduced functional valency of fetuin relative to ASF. At a concentration of 110 $\mu$M or greater, galectin-3 precipi-
did not substantially change by increasing the concentration of the galectin-3 from 130 to 240 μM.

**Electron Microscopy of the Galectin-3 Precipitates**—Fig. 5 shows the results of electron microscopy of the precipitates of galectin-3 with individual and mixed bivalent pentasaccharides. Large globular structures were observed in all the micrographs.

**DISCUSSION**

The present study shows that murine recombinant galectin-3 precipitates with a series of synthetic divalent carbohydrates possessing terminal LacNAc residues as well as ASF and fetuin. These latter results agree with Knibbs et al. (42), who also observed that galectin-3 precipitates with ASF and fetuin. These findings indicate that the lectin possesses multivalent carbohydrate binding and cross-linking activity. However, to understand the mechanism of binding and cross-linking of galectin-3, it is necessary to understand the relationship between the quaternary structure of the lectin and its precipitation activity. To directly determine the structure of murine recombinant galectin-3 in solution, we recently performed sedimentation velocity and equilibrium measurements of the protein (31). These measurements show that galectin-3 is predominantly a monomer in solution over the concentration range of 7–45 μM. However, a small increase in S20,w coupled with the decrease in D20,w in the sedimentation velocity experiments as a function of galectin-3 concentration suggests that the protein oligomerizes to a small extent with increasing concentrations. The S20,w and D20,w of the CRD domain of galectin-3 alone, however, does not change with increasing concentrations of protein, consistent with the hypothesis that the N-terminal domain is responsible for the association of galectin-3 in solution (25, 26) and not the C-terminal domain (43).

Similar sedimentation measurements of galectin-3 were performed in the presence of LacNAc (31), the nonreducing terminal disaccharide moiety in the pentasaccharides in Fig. 1 and ASF. These experiments addressed the possibility that binding of LacNAc itself could induce oligomerization of galectin-3. However, bound LacNAc did not change either the molecular mass or the hydrodynamic parameters of intact galectin-3 or its CRD domain (31). Therefore, galectin-3 does not oligomerize upon the binding of the LacNAc epitope.

**Precipitation Activity of Galectin-3**—The present results show that galectin-3 precipitates with ASF at a concentration as low as 10 μM lectin, indicating that an oligomeric form of galectin-3 must exist at this low concentration in addition to the predominant monomeric form of the protein. Fig. 3 also shows that galectin-3 at higher concentrations precipitates with the biantennary pentasaccharides in Fig. 1. This indicates the valency of the oligomeric form of galectin-3 must be greater than two because bivalent lectins do not precipitate with bivalent carbohydrates (44). Qualitative precipitation experiments with the pentasaccharides in Fig. 1 provides insight into the oligomeric form of the protein that precipitates with these carbohydrates.

Fig. 3 shows the quantitative precipitation profiles of galectin-3 in the presence of the 3,6-, 2,6-, and 4,6-pentasaccharides. Similar results were observed for the 2,4-pentasaccharide (not shown). The profiles of galectin-3 show single protein peaks in each experiment, indicating a single galectin-3 species precipitating with the pentasaccharides. The ratio of the galectin-3 monomer to bivalent saccharide at the point of maximum precipitation in the profiles ranges from 2.4:1 to 2.6:1 (Table I). The average percentage of precipitated galectin-3 is between 40 and 70% with the pentasaccharides (Table I). Because the structural valency of each pentasaccharide is 2, and two pentasaccharides (four equivalents of LacNAc) are minimally re-
quired to form a two-dimensional cross-linked complex (44), this suggests that galectin-3 precipitates as a pentamer (2.5:1) with the pentasaccharides. The proposed complex is shown in Scheme 1, which also indicates that one arm of the galectin-3 pentamer is not involved in cross-linking interactions. The ratio of 2:5 pentasaccharide/protein molecules does not rule out other higher ratios of the pentasaccharide to protein complexes but rather is the lowest ratio of carbohydrate to protein required for precipitation of a two-dimensional cross-linked complex.

Kinetics of Precipitation of Galectin-3 with Multivalent Carbohydrates and Model of a Pentamer-Monomer Equilibrium for the Lectin—The kinetics of precipitation of galectin-3 with ASF, fetuin, and the 3,6-pentasaccharide (Fig. 2) are all relatively fast, on the order of seconds. Because the precipitating species of galectin-3 appears to be a pentamer, the interconversion of monomer to pentamer must be fast to allow precipitation of the pentamer to be fast. Hence, the precipitating galectin-3 pentamer is rapidly replaced by monomer by mass action equilibria, as shown in Scheme 1. That this occurs by mass action equilibria and not a conformational change in the protein leading to oligomerization is supported by the fact that galectin-3 remains a monomer in the presence of LacNAc (31), the binding epitope(s) on the pentasaccharides in Fig. 1 and ASF. Importantly, precipitation of the cross-linked complexes of galectin-3 and phase transition from solution to solid phase of the lectin drives the mass action conversion of monomer to oligomer. (Interestingly, this may also occur upon binding of the galectin-3 oligomer to multivalent cell surface carbohydrate receptors, suggesting solid phase chemistry on the surface of cells.)

We also believe that there must be an equilibrium between two different conformations of monomeric galectin-3 (Scheme 1). One conformation would have the N-terminal domain tightly folded, which is consistent with the sedimentation data for galectin-3 (31). Another conformation of monomeric galectin-3 would exist with its N-terminal domain somewhat unfolded and directly involved in an equilibrium with the oligomeric form of the protein. The model in Scheme 1 is consistent with the observation that the N-terminal domain of galectin-3 is responsible for the oligomerization of the protein (25, 26). Importantly, the extended N-terminal domains of the oligomers interact with the carbohydrate ligands.

### Table I

| Pentasaccharide | Galectin-3 concentration | Buffer salt concentration | Peak ratio of galectin-3 to pentasaccharide | Galectin-3 precipitated at the peak |
|-----------------|--------------------------|---------------------------|---------------------------------------------|-----------------------------------|
| 2,6-Penta       | 150                      | 0.15                      | 2.6:1                                       | 45                                |
| 3,6-Penta       | 110                      | 0.15                      | 2.6:1                                       | 49                                |
| 3,6-Penta       | 110                      | 1.0                       | 2.4:1                                       | 64                                |
| 3,6-Penta       | 130                      | 0.15                      | 2.5:1                                       | 71                                |
| 4,6-Penta       | 253                      | 0.15                      | 2.5:1                                       | 48                                |
| (2,6 + 3,6)-Penta | 150                   | 0.15                      | 2.5:1                                       | 47                                |
| (2,6 + 4,6)-Penta | 150                  | 0.15                      | 2.5:1                                       | 58                                |
| (3,6 + 4,6)-Penta | 130                  | 0.15                      | 2.5:1                                       | 34                                |
| (3,6 + 4,6)-Penta | 240                  | 0.15                      | 2.4:1                                       | 55                                |
Gomer of galectin-3 are likely to be flexible, making the overall oligomer highly flexible.

Scheme 1 also explains the reports of positive binding cooperativity of galectin-3 (30). The addition of unlabeled galectin-3 to 125I-labeled galectin-3 is reported to increase binding of the labeled lectin to immobilized laminin, a large extracellular matrix glycoprotein containing polylactosamine chains. The addition of unlabeled galectin-3 would increase the amount of oligomeric galectin-3 in solution by the equilibria shown in Scheme 1. This would result in an increase in the amount of 125I-labeled galectin-3 oligomer, which in turn would further bind to laminin. Hence, the addition of unlabeled galectin-3 would result in the apparent positive binding cooperativity of the labeled lectin according to the equilibria in Scheme 1.

**Mixed Quantitative Precipitation Experiments and Electron Microscopy Results:** Evidence for the Formation of Heterogeneous Cross-linked Complexes by Galectin-3

A remaining question is whether galectin-3 forms homogeneous or heterogeneous cross-linked complexes with the pentasaccharides in Fig. 1. Mixed quantitative precipitation experiments have been used to determine whether lectins form homogeneous cross-linked lattices with multivalent carbohydrates possessing specific epitopes (45). The presence of two protein peaks in the precipitation profile of a single lectin mixed with two multivalent carbohydrates is evidence for the formation of homogeneous cross-linked complexes of the lectin with each carbohydrate (44).

Mixed quantitative precipitation profiles of galectin-3 with binary mixtures of the pentasaccharides in Fig. 1 were therefore determined. Galectin-3 was mixed with 1:1 molar ratios of the 2,6- and 3,6-pentasaccharides, the 2,6- and 4,6-pentasaccharides, and the 3,6- and 4,6-pentasaccharides. The resulting precipitation profiles are shown in Fig. 4. Each profile shows a single protein peak similar to that observed for the individual pentasaccharides (Fig. 3). The ratio of galectin-3 monomer to total sugar concentration at the point of maximum precipitation is also similar to the range observed for single pentasaccharide experiments.

The presence of a single peak rather than two protein peaks in the mixed quantitative precipitation profiles of galectin-3 with binary mixtures of the pentasaccharides suggests that galectin-3 does not form homogeneous cross-linked lattices with these bivalent oligosaccharides. Instead, the similarity of the precipitation data for the mixtures of pentasaccharides with that of the individual carbohydrates (Table I) suggests that galectin-3 forms heterogeneous cross-linked complexes with these oligosaccharides. This is shown in Scheme 2, in which a single galectin-3 pentamer is involved in binding and cross-linking with two different pentasaccharides.

Negative stain electron microscopy experiments of the precipitates of galectin-3 with single pentasaccharides or mixtures of the pentasaccharides also fail to reveal structures associated with the respective precipitates (Fig. 5). Instead, large globules are observed. These results are in contrast to the electron microscopy patterns observed for the precipitates of the soybean agglutinin with the individual pentasaccharides in Fig. 1 (46) as well as isolecitin A of Lotus tetragonolobus with several fucosyl oligosaccharides (47). The present electron microscopy results in Fig. 5 are thus consistent with galectin-3 forming disorganized, heterogeneous cross-linked complexes (Scheme 3B), as opposed to organized, homogeneous cross-linked complexes (Scheme 3A).

**Biological Implications**—Galectin-3 is unique in its anti-apoptotic activity as compared with other galectins, including galectin-1, that possess apoptotic activities (48). In addition, galectin-3 antagonizes the biological effects of galectin-1 at the level of ligand binding in two cell systems: the binding of galectin-1 to susceptible T cells leading to apoptosis (49) and the growth inhibition effects of galectin-1 binding to a neuroblastoma cell line (50). The results of the present study show that differences in the quaternary structure and carbohydrate cross-linking activity may account for these inhibitory effects of galectin-3 on galectin-1, as well as their opposing activities in other biological systems. The present results may also have implications for understanding the complex growth regulatory network of galectins in tumor cells (51). A comparison of the quaternary structures and carbohydrate cross-linking activities of galectin-1 and -3 is given below.

Galectin-1 is a prototypic member of the galectin family that

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a L. Baum, private communication.
possesses one CRD domain and exists predominantly as a noncovalent dimer in solution in the presence and absence of LacNAc (31). Galectin-1 also remains a dimer in cross-linked complexes with a bivalent oligosaccharide (8). Galectin-3, on the other hand, is predominantly a monomer in solution but rapidly converts to a pentamer by mass action equilibria in the presence of a precipitating multivalent carbohydrate (Scheme 1). Hence, galectin-3 is converted to a larger oligomer (pentamer) than galectin-1 (dimer) in the presence of certain multivalent carbohydrates.

Importantly, the type of cross-linked complexes formed by galectin-1 and -3 with multivalent carbohydrates are different. Galectin-1 can form linear type 1 (one-dimensional) cross-linked complexes (52) with bivalent carbohydrates (8) that can possess a high degree of polymorphism (8). Galectin-1 can also form type 2 (two- and three-dimensional) homogeneous cross-linked complexes with multivalent carbohydrates that possess valencies greater than two (52). These cross-linked lattices are highly organized and unique for each lectin-carbohydrate pair (Scheme 3A). On the other hand, the present study indicates that galectin-3 forms type 2 heterogeneous cross-linked complexes with multivalent carbohydrates (Scheme 3B). Such complexes are structurally disorganized in contrast to type 2 homogeneous cross-linked complexes.

These differences in the quaternary structures and cross-linking activities can account for some of the inhibitory effects of galectin-3 on the activities of galectin-1 in two cell systems. The observation that exogenously added galectin-3 antagonizes the apoptotic effects of galectin-1 in susceptible T cells (49) can be understood in terms of the greater avidity of the galectin-3 pentamer as compared with the galectin-1 dimer for glycoprotein receptors on the cells. This is especially true if the carbohydrate moieties of the receptors involve LacNAc residues because the affinities of both proteins are nearly the same toward this epitope (36). Hence, galectin-3 would be expected to competitively displace galectin-1 from the cells. In addition, galectin-1 binding results in the redistribution of specific galectin-1 counter-receptors by galectin-1 and galectin-3, respectively, also applies to the immunoregulatory effects of these two lectins on T-cell receptor organization.

Summary—The results of the present study provide a model for the carbohydrate binding and cross-linking properties of galectin-3. The unique chimeric structure of galectin-3 contributes to its ability to dynamically convert to pentamers that form heterogeneous type 2 cross-linked complexes with specific multivalent carbohydrates (Schemes 2 and 3B). This contrasts with galectin-1 (and presumably other members of the galectin family with static divalent structures), which can form homogeneous type 2 cross-linked complexes with multivalent carbohydrates (Scheme 3A). The biological consequences of these opposing cross-linking activities, along with their different quaternary structures, may explain in part some of the anti-apoptotic activities of galectin-3 and apoptotic activities of galectin-1. The theme of actively organizing or disorganizing counter-receptors may be a general theme among the galectins and in related biological systems.

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Multivalent Cross-linking Studies of Galectin-3
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