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Multivalent Protein−Carbohydrate Interactions. A New Paradigm for Supermolecular Assembly and Signal Transduction

James C. Sacchettini,[‡] Linda G. Baum,[§] and C. Fred Brewer*,[||]

Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843, Department of Pathology, University of California, Los Angeles, California 90095, and Departments of Molecular Pharmacology and of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: Many biological recognition processes involve the binding and clustering of ligand−receptor complexes and concomitant signal transduction events. Such interactions have recently been observed in human T cells in which binding and cross-linking of specific glycoprotein counter-receptors on the surface of the cells by an endogenous bivalent carbohydrate binding protein (galectin-1) leads to apoptosis [Pace, K. E., et al. (1999) *J. Immunol. 163*, 3801−3811]. Importantly, different counter-receptors associated with specific phosphatase or kinase activities were shown to form separate clusters on the surface of the cells as a result of galectin-1 binding to the carbohydrate moieties of the respective glycoproteins. This suggests that the unique separation and organization of signaling molecules that results from galectin-1 binding is involved in delivering the signal to die. The ability of galectin-1 to induce the separation of specific glycoprotein receptors was modeled on the basis of molecular and structural studies of the binding of multivalent carbohydrates to lectins that result in the formation of specific two- and three-dimensional cross-linked lattices. These latter studies have been recently highlighted by X-ray crystallographic results showing that a single tetravalent lectin forms distinct cross-linked complexes with four different bivalent oligosaccharides [Olsen, L. R., et al. (1997) *Biochemistry 36*, 15073−15080]. In this report, binding and cross-linking of multivalent carbohydrates with multivalent lectins is shown to be a new paradigm for supermolecular assembly and signal transduction in biological systems.

A large number of biological processes involve multivalent binding interactions, including cellular adhesion and signal transduction events (*1*). Considerable attention has focused on bivalent−bivalent interactions, since many ligand−receptor systems, including growth factors, appear to induce dimerization of their receptors, leading to signal transduction (*2*). However, other systems involve higher degrees of multivalent interactions, including the binding of multivalent carbohydrates and glycoconjugates with lectins. Molecular and structural studies have shown that cross-linking interactions between certain multivalent lectins and glycoconjugates result in the formation of unique two- and three-dimensional supermolecular assemblies (*3*, *4*). Recently, such assemblies have been invoked to describe the clustering and separation of different glycoprotein receptors on the surface of human T cells induced by a single endogenous lectin (galectin-1)

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* To whom correspondence should be addressed.
[‡] Texas A&M University.
[§] University of California.
[||] Albert Einstein College of Medicine.

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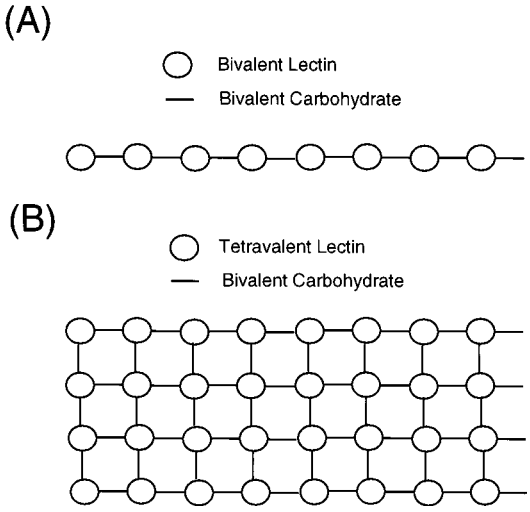

FIGURE 1: (A) Schematic diagram of a type 1 cross-linked complex between a bivalent lectin and a bivalent carbohydrate. (B) Schematic diagram of a type 2 cross-linked complex between a tetravalent lectin and a bivalent carbohydrate. The lectin is represented by a circle and the carbohydrate by a line in both schemes.

which leads to apoptosis (*5*). In the report presented here, recent structural studies of the molecular assembly of multivalent lectin−carbohydrate cross-linked complexes are reviewed and discussed in terms of the galectin-1-mediated apoptotic signal in human T cells.

Lectins are carbohydrate binding proteins that are widely conserved in nature, including in animals, plants, and microorganisms (*6*). The biological roles of many animal lectins are known. For example, a number of mammalian lectins are involved in receptor-mediated endocytosis of glycoproteins, and others, including the selectins, are involved in cellular recognition and adhesion (*7*). Certain members of the galectin family have been implicated in metastasis, in control of cell growth, in activation of inflammatory cells, and in regulation of apoptosis (*8*).

A common feature of lectins is their multivalent binding activities. The X-ray crystal structures of many plant (*9*) and animal lectins (*10*) demonstrate their oligomeric structures. As a consequence, lectin binding to cells often leads to cross-linking and aggregation of specific glycoprotein and glycolipid receptors. In many cases, these cross-linking interactions are associated with signal transduction effects, including the mitogenic activities of lectins (*11*), the induction of mating reactions in fungi (*12*), and the induction of apoptosis in activated human T cells (*5*, *13*).

Recent structural studies of multivalent lectin−carbohydrate cross-linked complexes have provided new insights into the specificity of these interactions. In general, two types of multivalent complexes are observed, designated type 1 and type 2 (*3*). In type 1 complexes, cross-linking occurs between a bivalent carbohydrate and bivalent lectin. Figure 1A shows a schematic diagram of type 1 interactions, which may result in the formation of linear (one-dimensional) cross-linked complexes. These complexes are often soluble (*14*, *15*) and possess a high degree of polymorphism due to their flexibility. In theory, such complexes may accommodate more than one type of bivalent lectin or bivalent carbohydrate in the cross-linked complex. Examples include the X-ray crystal complexes of dimeric galectin-1 from bovine heart muscle cross-linked with a bivalent N-linked biantennary complex

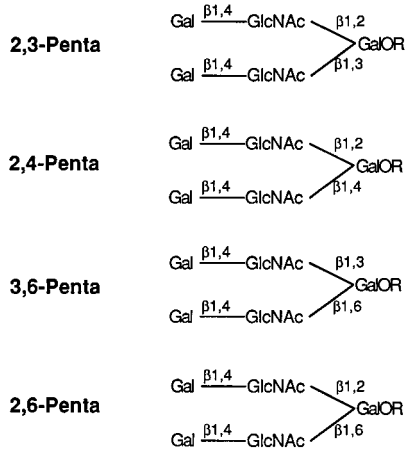

FIGURE 2: Structures of the 2,3-, 2,4-, 3,6-, and 2,6-pentasaccharides. The aglycon moiety R is $(CH_2)_5COOCH_3$. The core galactose residue is in the β-anomeric configuration.

glycopeptide (*16*). Three different crystal structures are observed for the galectin cross-linked with three different conformations of the glycopeptide. These findings emphasize the structural flexibility of type 1 interactions.

In type 2 complexes, cross-linking occurs between lectins and carbohydrates in which one of the two molecules possesses a valency of >2. An example is a cross-linked complex between a tetravalent lectin and a bivalent carbohydrate as shown schematically in Figure 1B. Such interactions lead to the formation of two- and three-dimensional cross-linked complexes, which can be insoluble and precipitate from solution (*17*−*19*). Type 2 complexes may exist as ordered cross-linked lattices, as illustrated in Figure 1B. These interactions have been termed homogeneous cross-linking, since a given lattice consists of a single carbohydrate and a single lectin, even in the presence of multiple carbohydrates and lectins in solution (*3*).

Until recently, the molecular basis for the formation of these ordered carbohydrate−lectin type 2 lattices was poorly understood. However, the X-ray crystal structures of a single tetravalent lectin cross-linked with four different bivalent carbohydrates have recently been reported (*20*, *21*). These results have provided insight into the molecular basis for the formation of type 2 homogeneous carbohydrate−lectin noncovalent cross-linked complexes.

X-ray Structures of SBA[1] Cross-Linked with Four Isomeric Bivalent Pentasaccharides

The soybean agglutinin (SBA) is a tetrameric lectin with binding specificity for GalNAc and Gal residues. Previous quantitative precipitation experiments and electron micrograph studies (*22*) suggested that SBA formed unique type 2 cross-linked complexes with the four bivalent pentasaccharides shown in Figure 2. The X-ray structure of SBA cross-linked with the 2,6-pentasaccharide was subsequently reported (*20*). The structure of the complex was determined by molecular replacement techniques and refined at 2.6 Å resolution to an *R* value of 20.1%. The crystals were

[1] Abbreviations: SBA, lectin from soybeans (*Glycine max*); ConA, lectin from jack beans (*Canavalia ensiformis*); N-linked, asparagine-linked; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; LacNAc, Galb(1,4)GlcNAc; NMR, nuclear magnetic resonance; EM, electron microscopy. All sugars are in the D-configuration.

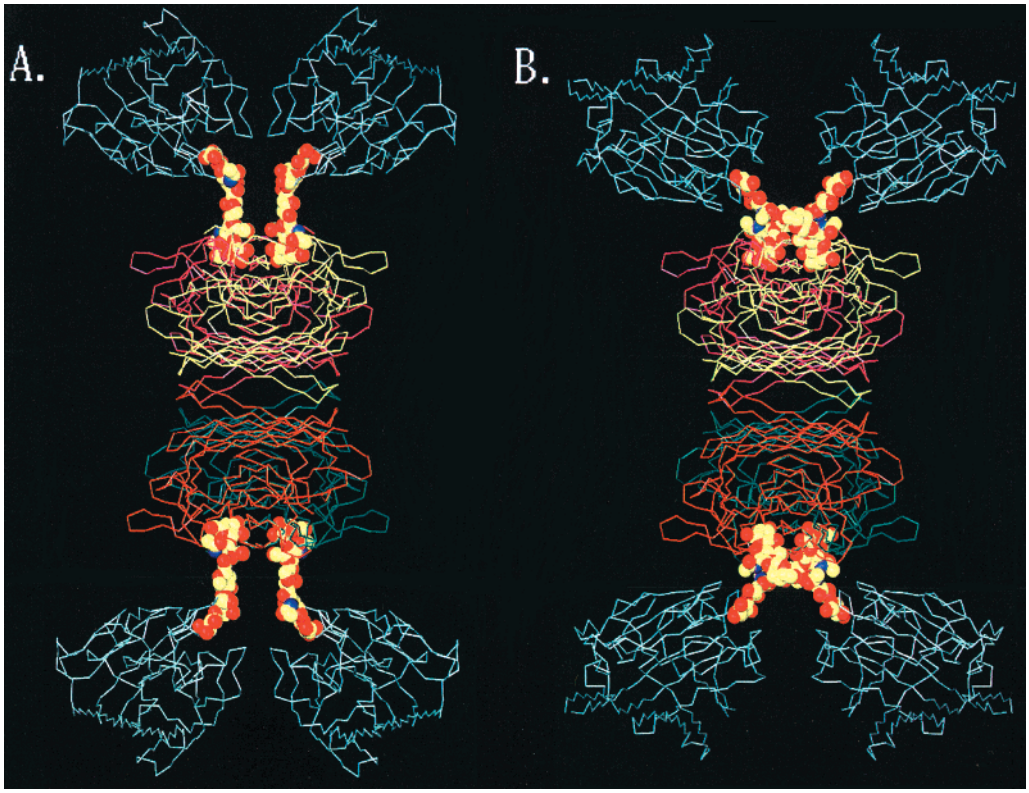

FIGURE 3: Structures of (A) the 3,6-pentasaccharide cross-linked lattice with SBA and (B) the 2,3-pentasaccharide cross-linked lattice with SBA (*21*). The views are in the *z* plane perpendicular to a 2-fold axis of the tetramer. Protein molecules are shown as trace models and the cross-linking oligosaccharides as CPK models (the aglycon moiety is not shown). One SBA tetramer in the middle of each structure (shown as yellow, magenta, green, and red trace models) is cross-linked by four pentasaccharide molecules to four SBA monomers (shown in aqua) which are part of adjacent tetramers (not shown). One arm of a pentasaccharide molecule binds to the carbohydrate binding site on each subunit of a tetramer and cross-links to a neighboring tetramer by binding to the corresponding site on another monomer. These figures were generated with the program O.

hexagonal in space group $P6_422$, which differs significantly from that of crystals of the free protein (space group *C*2). Analysis of the crystal lattice revealed that the structural basis of the complex was generated by pentasaccharide molecules cross-linking neighboring SBA subunits. Association between SBA tetramers involved binding of the terminal Gal residues of the pentasaccharide at identical sites in each monomer, with the oligosaccharide cross-linking to a symmetry-related neighbor molecule.

More recently, the X-ray crystal structures of SBA cross-linked with the 2,3-, 2,4-, and 3,6-pentasaccharides in Figure 2 were reported (*20*, *21*). Examination of the protein structures of SBA in the cross-linked complexes with the 2,3-, 2,4-, 2,6-, and 3,6-pentasaccharides shows that they are similar despite differences in the crystal lattice of the complexes (*20*). This indicates that differences in the lattice are dictated by the structures of the cross-linking carbohydrates. The structures of a portion of the 3,6-pentasaccharide cross-linked lattice with SBA and the 2,3-pentasaccharide cross-linked complex with SBA are shown in panels A and B of Figure 3, respectively. It can be seen that the structure and conformation of the bound carbohydrates are different in the two complexes, and as a consequence, the spacings between outer cross-linked SBA molecules (shown as monomers) are different in the two complexes.

Table 1 shows the space group and unit cell dimensions of all four cross-linked complexes (*21*). While the 2,4-, 3,6-, and 2,6-pentasaccharide−SBA lattices belong to the same space group ($P6_422$), they have distinctly different unit cell

Table 1: Space Group and Unit Cell Parameters of SBA Cross-Linked with the 2,3-, 2,4-, 3,6-, and 2,6-Pentasaccharides

complex	space group	*a* (Å)	*b* (Å)	*c* (Å)	α (deg)	*β* (deg)	*γ* (deg)
2,3-SBA	I_122	122.6	122.6	90.6	90	90	90
2,4-SBA	$P6_422$	144.6	144.6	107.2	90	90	120
3,6-SBA	$P6_422$	143.3	143.3	107.8	90	90	120
2,6-SBA	$P6_422$	144.9	144.9	109.4	90	90	120

dimensions. The 2,4- and 2,6-pentasaccharide lattices exhibit small differences (0.3 Å) along their *a* and *b* axes, with a larger difference (2.2 Å) along the *c* axes. The 3,6-pentasaccharide lattice differs from the other two lattices along the *a* and *b* axes (1.3−1.6 Å). The lattice of the 3,6-pentasaccharide−SBA complex also differs along the *c* axis by 0.6 Å relative to the lattice of the 2,4-pentasaccharide−SBA complex. Differences in the *c* axes of all three lattices can be related to the distances separating the C-6 carbon atoms of the terminal Gal residues of each cross-linking pentasaccharide in symmetry-related SBA monomers. The distances of 19.2, 19.7, and 20.6 Å that separate the C-6 atoms of the terminal Gal residues of the 2,4-, 3,6-, and 2,6-pentasaccharides, respectively, correlate with the order of the distances along the *c* axes for all three complexes of 107.2, 107.8, and 109.4 Å (Table 1), respectively. Thus, the *c* axes of the three complexes reflect differences in the lengths of the three oligosaccharides.

In the case of the 2,3-pentasaccharide, the distance between the C-6 atoms of the terminal Gal residues is 15.0 Å, which

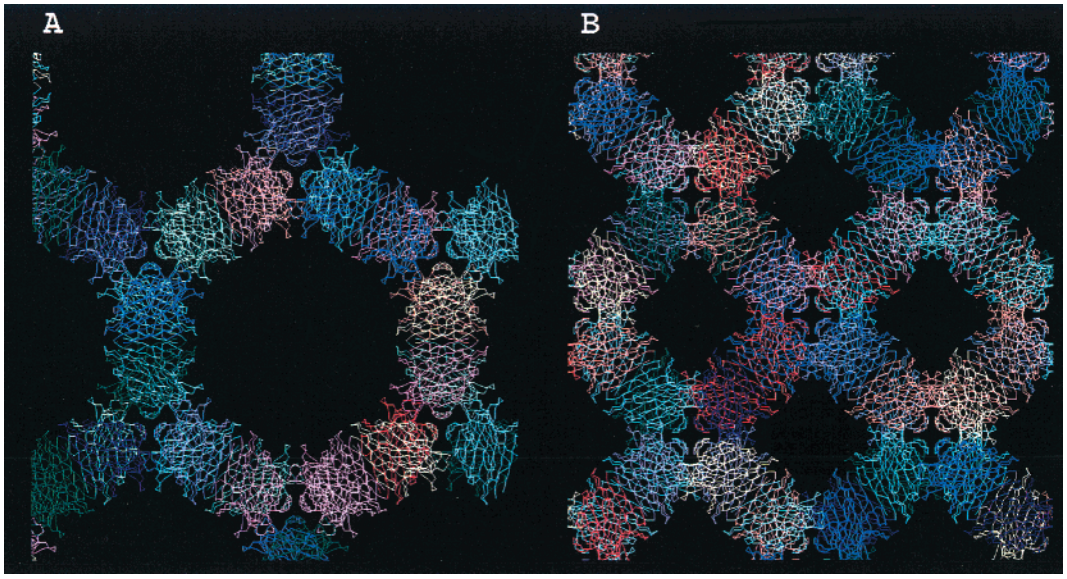

FIGURE 4: (A) Six-fold axis of symmetry down the *c* axis shown for the 3,6-pentasaccharide cross-linked complex with SBA and (B) 4-fold axis of symmetry down the *c* axis shown for the 2,3-pentasaccharide cross-linked complex with SBA (*21*). In these views, protein molecules are shown as colored trace models (monomers), with the cross-linking oligosaccharides not shown. These figures were generated with MOLPACK.

is considerably shorter than the respective distances of the other three carbohydrates. More importantly, the angle between the two LacNAc arms is sufficiently acute to cause the lattice to adopt a different space group ($I_1$22). The crystal lattices of the 2,4-, 3,6-, and 2,6-pentasaccharide−SBA complexes possess a common 6-fold axis of symmetry. The 6-fold axis of symmetry down the *c* axis for the 3,6-pentasaccharide cross-linked complex with SBA is shown in Figure 4A. A salient feature of the lattice(s) is the absence of a high degree of strong protein−protein contacts, and formation of large, solvent-filled cavities (∼70%). By comparison, the 2,3-pentasaccharide complex exhibits a 4-fold axis of symmetry, which is shown in Figure 4B. The 4-fold axis of symmetry results in a cavity between lectin molecules with a diameter of ∼45 Å, as compared to ∼100 Å for the other three lattices.

These results demonstrate that the molecular bases for the formation of homogeneous type 2 carbohydrate-protein cross-linked complexes between SBA and the four carbohydrates in Figure 2 are the differences in the atomic structures of the oligosaccharides and their effects on the crystal packing interactions in each complex. These interactions are made possible by the pseudosymmetry of the outer carbohydrate epitopes of the pentasaccharides shown in Figure 2, which are often found in other naturally occurring branch chain carbohydrates (*22*), and the symmetry of the carbohydrate binding sites of the lectin (*20*).

The above examples of SBA cross-linked with bivalent carbohydrates result in the formation of three-dimensional cross-linked complexes. Our laboratory has also shown that a two-dimensional cross-linked complex forms between the fucose-specific lectin from *Lotus tetragonolobus*, isolectinA, and a bivalent fucosyl oligosaccharide (*23*). This two-dimensional type 2 cross-linked complex may be more likely to occur on the surface of cells.

Formation of Homogeneous Glycoprotein−Lectin Cross-Linked Complexes

Evidence that lectins also form organized cross-linked complexes with glycoproteins exists. Using quantitative precipitation analysis, the Man/Glc-specific jack bean lectin concanavalin A (ConA) was shown to bind and form unique, stoichiometric cross-linked complexes with four different glycoproteins which possess different numbers and types of carbohydrate chains as well as different quaternary structures (*18*). All of the glycoproteins possess N-linked carbohydrates that bind to ConA. The unique binding stoichiometries of ConA with each glycoprotein were observed to be maintained in the presence of binary mixtures of the glycoproteins. These results were taken as evidence of the formation of homogeneous cross-linked complexes between ConA and each glycoprotein.

Similar studies have shown that galectin-1 from calf spleen, a dimeric animal lectin, and several Gal-specific plant lectins form specific, stoichiometric cross-linked complexes with asialofetuin (ASF), a 48 kDa monomeric glycoprotein possessing three triantennary N-linked complex carbohydrates with terminal Gal residues (*19*). The unique binding stoichiometries of calf spleen galectin-1 with ASF were also observed in the presence of binary mixtures of the lectins and ASF. These results were evidence of the formation of homogeneous cross-linked complexes between calf spleen galectin-1 and ASF, as well as the other Gal-specific lectins and ASF, even in the presence of mixtures of the molecules. Studies with the 16 kDa galectin from chicken liver also produced unique binding stoichiometries with ASF similar to that of galectin-1 from calf spleen (*24*). Interestingly, a natural lactose-binding immunoglobin G fraction from human serum also exhibited a unique binding stoichiometry with ASF (*24*), suggesting the possibility of ordered cross-linked complexes between the antibodies and the glycoprotein.

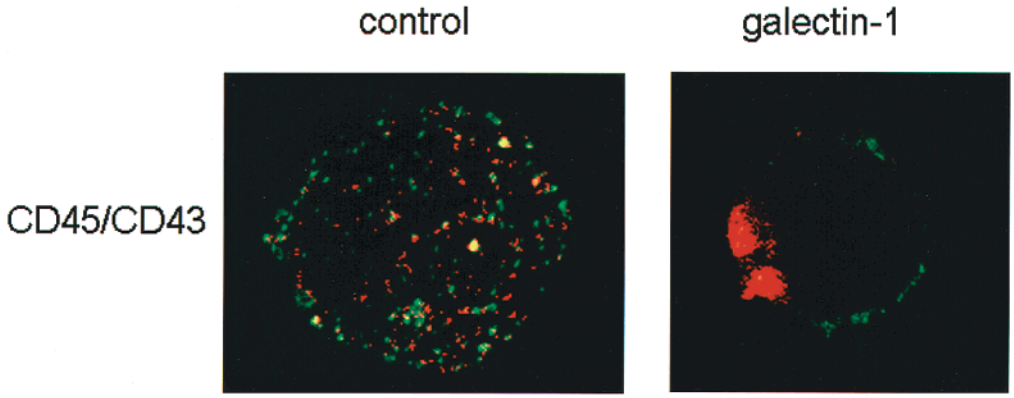

FIGURE 5: Confocal micrographs (100×) of MOLT-4 cells before and after exposure to galectin-1 (*5*). After galectin-1 treatment, CD45 localized into one or two large islands and CD43 clustered into multiple small aggregates. Control panel (no galectin-1): uniform distribution of CD45 (red) and CD43 (green) on the surface of MOLT-4 cells with some areas of colocalization (yellow). Views are of 0.5 mm slices through the top (left) or center (right) of a single cell. Reprinted with permission from the *Journal of Immunology*.

Restricted Receptor Segregation into Membrane Microdomains Occurs on Human T Cells during Apoptosis Induced by Galectin-1

Apoptosis is a critical process regulating T cell development in the thymus and in controlling the immune response in the periphery. Galectin-1 induces apoptosis of human thymocytes, activated T cells, and T lymphoblastoid cell lines (*5*). In the thymus, galectin-1 is produced by thymic epithelial cells. Elucidation of the mechanism of galectin-1-induced apoptosis of susceptible thymocytes is thus critical for understanding the role of galectin-1 in immune development and in the immune response. In addition, the induction of T cell apoptosis is one of the first signaling functions identified for a mammalian lectin.

The dimeric form of galectin-1 is required for induction of apoptosis in susceptible T cells (*13*), implying that cross-linking of cell surface receptors is involved in transducing the apoptotic signal. The addition of lactose was also observed to inhibit apoptosis, demonstrating that galectin-1 binding to the carbohydrate chains of the glycoprotein receptors is involved in the cross-linking mechanism. Importantly, the initial steps in galectin-1 binding and signaling were shown to involve segregation of specific T cell surface glycoprotein receptors into discrete membrane microdomains (*5*). Several T cell glycoproteins from MOLT-4 human T cells were demonstrated to be specific receptors for mammalian galectin-1 binding: CD45, CD43, and CD7 (*5*). Previous studies with individual deletion mutants of the glycoproteins confirmed their importance in the apoptotic response of the cells to galectin-1 (*5*, *13*, *25*; J. T. Nguyen and L. G. Baum, unpublished data). Other T cell glycoproteins have also been shown to bind galectin-1, including CD2, CD3, and CD4, although these receptors are not required for cell death (*5*, *25*).

Figure 5 shows immunofluorescent localization studies, performed by confocal microscopy, designed to assess the distribution of CD45 and CD43 receptors on the surface of MOLT-4 human T cells in the absence (control) and presence of galectin-1 after 20 min at 37 °C (*5*). Exposure of the cells to galectin-1 for this period of time was sufficient to initiate the apoptotic pathway. Before addition of galectin-1 (control), the distribution of CD45 and CD43 is observed to be nearly random across the surface of the cells [antibodies to CD45 are labeled with Texas Red (red) and antibodies to CD43 are labeled with fluorescein (green)]. After 20 min, the cells were cooled to prevent progression along the death pathway, galectin-1 was removed with lactose, and the cells were treated with paraformaldehyde to fix membrane glycoproteins. Galectin-1 binding resulted in a dramatic redistribution of the receptors with CD45 localized on two large patches that were shown to be apoptotic membrane blebs. CD43 clustered into smaller patches that were segregated from CD45. Thus, binding of galectin-1 to T cells induced redistributuion and segregation of CD45 and CD43, suggesting that this may be important for sending the apoptotic signal.

In a similar experiment, galectin-1 binding was shown to result in the clustering of CD7 with CD43, segregated from CD45, as shown in Figure 5 for CD43 (*5*). CD7 and CD43 were colocalized prior to galectin-1 addition, but distributed diffusely over the cell surface. Galectin-1 binding caused the aggregation of CD7−CD43 complexes. Additional studies also showed that CD3 colocalized with CD45 following galectin-1 treatment (*5*). Thus, CD45−CD3 and CD43−CD7 complexes were demonstrated to separate from each other into distinct patches as observed in Figure 3 for CD45 and CD43.

The physical separation of the CD45−CD3 complex from the CD43−CD7 complex induced by galectin-1 binding and cross-linking of the carbohydrate chains of the respective glycoproteins has been modeled on the basis of the lattice model for type 2 lectin−carbohydrate cross-linking interactions (Figure 1) (*3*). A schematic of these interactions is shown in Figure 6 (*5*). Galectin-1 is envisioned as binding to the carbohydrate chains of CD45 and inducing cross-linking and separation of the CD45−CD3 complex. Likewise, galectin-1 is envisioned to bind to the carbohydrate chains of the CD43−CD7 complex and cross-link this binary complex into a separate lattice or series of lattices. The fact that binary complexes of CD45 with CD3 and CD43 with CD7 are observed to separate in the presence of galectin-1 suggests that CD45 and CD3 may bind to each other by protein−protein interactions as do CD43 and CD7. The fact that both complexes separate together further suggests that each binary complex behaves as a single molecular species with covalently attached carbohydrate that mediates cross-linking with galectin-1. Thus, unlike the in vitro experiments by Brewer and co-workers who observed individual glyco-

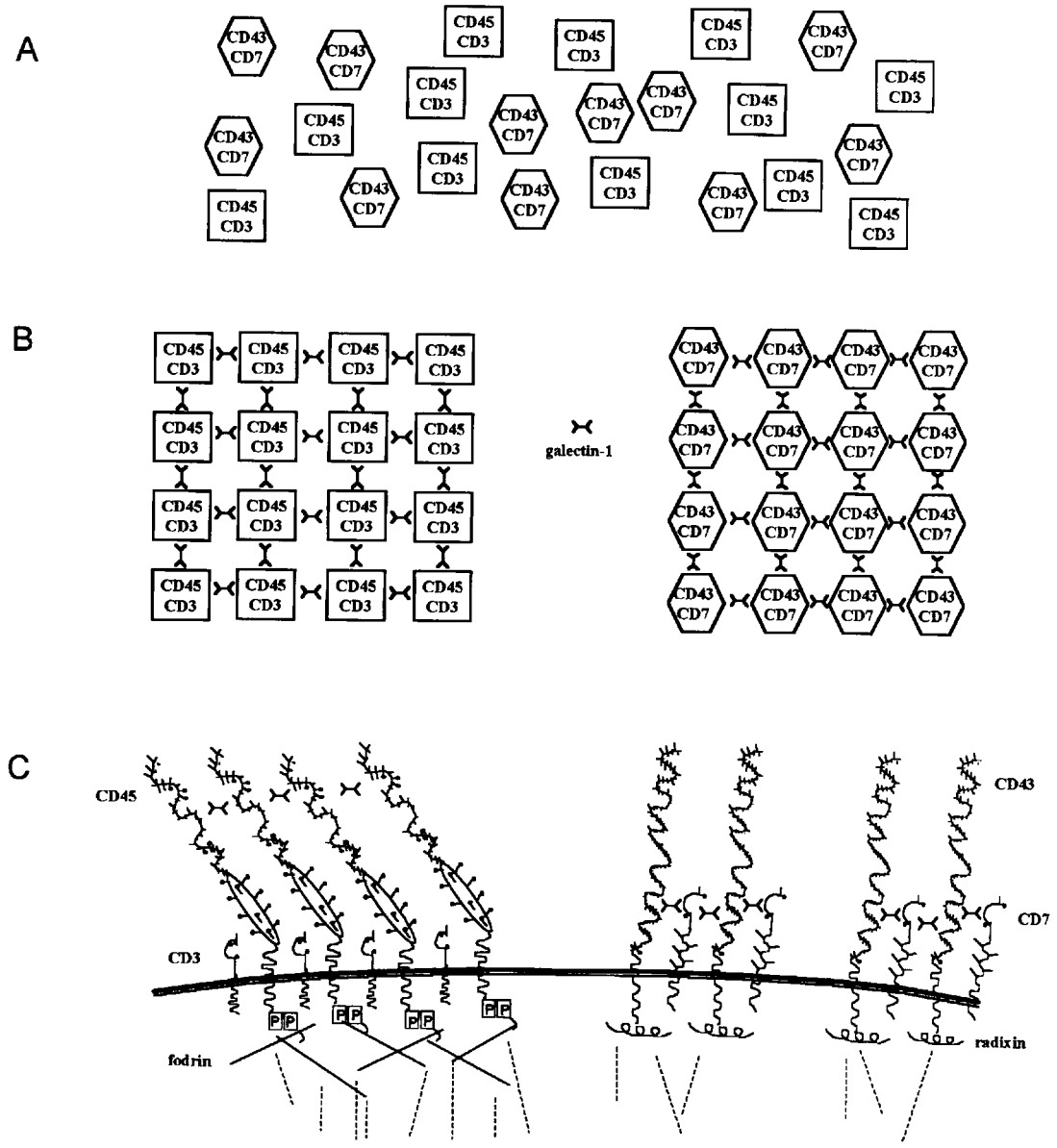

FIGURE 6: Schematic representation of the redistribution and segregation of galectin-1 receptors following binding of galectin-1 (*5*). (A) Random distribution of CD45 and CD3 complexes (squares) and CD43 and CD7 complexes (hexagons) on the cell surface, in the absence of galectin-1. (B) Galectin-1 binding results in homotypic lattice formation of repeating units of the CD45 and CD3 complexes (squares) and CD43 and CD7 complexes (hexagons). (C) Cross-linked complexes of galectin-1 receptors with associated cytoskeletal proteins. Reprinted with permission from the *Journal of Immunology*.

proteins forming organized cross-linked complexes with a given lectin (*3*), the observations of Baum and co-workers of binary complexes of glycoproteins that separate together in the presence of a single lectin (galectin-1) require tight binding of CD45 to CD3 and CD43 to CD7 to present themselves as unit masses capable of undergoing organized lattice formation via galectin-1-induced cross-linking of their respective carbohydrate chains.

How would cross-linking lattices of glycoproteins on the cell surface trigger an apoptotic pathway? The cytoplasmic tail of CD45 has two protein tyrosine phosphatase domains (*26*). CD43 has no known enzymatic activity, but associates with the *fyn* tyrosine kinase. Similarly, CD7 has no enzymatic activity, but ligation of CD7 results in tyrosine phosphorylation (*27*), as does galectin-1 binding (*28*). While the requirement for tyrosine phosphorylation in galectin-1-induced apoptosis is not known, this suggests that the segregation of the phosphatase activity of CD45 from the kinase activities associated with the CD43−CD7 complex may facilitate transmission of a death signal via tyrosine phosphorylation.

Other Multivalent Molecules and Systems

Since the formation of homogeneous lectin−carbohydrate cross-linked complexes is a thermodynamically driven process, other multivalent ligand−receptor systems may also undergo similar selective cross-linking interactions. These include antibody−antigen or antibody−receptor interactions (*29*), MHC−T cell receptor interactions (*30*, *31*), protein−lipid and lipid−lipid interactions (*32*), and egg−sperm interactions (*33*). The presence of lectins in trafficking and secretory pathways is also well-known (*34*). Many of these lectins may function by selectively cross-linking and separating specific multivalent glycoproteins.

Importantly, not all multivalent ligand−receptor interactions lead to the formation of homogeneous cross-linked

complexes, and limitations exist in the structures of multivalent molecules that undergo these interactions (*3*). Separation of the binding epitopes or domains of multivalent molecules by spacer groups can lead to the formation of amorphous cross-linked complexes, or specific heterogeneous cross-linked complexes which may involve three or more types of multivalent molecules. Examples of homogeneous versus heterogeneous and amorphous cross-linking interactions are the family of dimeric F-actin cross-linking proteins that are found in actin bundles and networks (*35*). Tandom domain dimeric F-actin cross-linking proteins form actin bundles which are tightly spaced together (homogeneous cross-linking), while certain dimeric F-actin cross-linking proteins with spacer groups between the binding domains form amorphous or heterogeneous cross-linked complexes with F-actin (*3*). The structures of a wide variety of animal lectins also follow these structural themes (*36*), suggesting that different lectins may form homogeneous or heterogeneous cross-linked complexes with specific glycoconjugates.

Summary

Studies of the binding and cross-linking interactions of multivalent carbohyrdrates with lectins present a model for lectin−glycoconjugate multidimensional clustering in vivo and a general thermodynamic mechanism for selectively aggregating a dispersed population of multivalent molecules and receptors in biological systems. These types of interactions may be of particular importance in separating kinase and phosphatase activities from each other in signal transduction mechanisms, and in the separation of similar classes of molecules (glycoproteins) from each other in transport processes.

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