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13. ABSTRACT *(Maximum 200 Words)*

Tumor cell resistance to apoptosis is a major obstacle to effective therapy of prostate cancer. We have found that the androgen dependent prostate cancer cell line LNCaP is sensitive to apoptosis induced by galectin-1, an endogenous human lectin that is abundant in prostate stroma. In contrast, androgen independent LNCaP, DU145 and PC3 cells are resistant to galectin-1 induced death and actually synthesize galectin-1 and export it to the cell surface. Galectin-1 binds to saccharide ligands on susceptible LNCaP cells to trigger cell death. Susceptibility to galectin-1 appears to depend on the presence of a specific class of cell surface glycans, the O-linked glycans on glycoproteins; in contrast, N-glycans are not required for galectin-1 induced LNCaP cell death. Resistance to galectin-1 induced death correlates with markedly decreased expression of a specific glycosyltransferase, the C2GnT, which creates saccharide ligands on O-glycans that are recognized by galectin-1. The C2GnT enzyme also regulates susceptibility of T cells to galectin-1 induced death, indicating that a common glycosylation pathway may control cell death in epithelial and lymphoid cells. Identification of a mechanism that enhances galectin-1 prostate cancer cell death may allow novel therapeutic approaches to manipulate tumor cell glycosylation to overcome tumor cell resistance to apoptosis.

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Appendix 1: Brewer CF, MC Miceli and **Baum LG** (2002) Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide mediated cellular interactions. *Current Opinion in Structural Biology* 12:616-623

Introduction

Regulating the life and death of tumor cells is an important goal in controlling the spread of cancer. We are examining apoptosis, or programmed cell death, induced by a human lectin termed galectin-1. The galectins are a family of mammalian lectins, or carbohydrate binding proteins, that have multiple functions, including positive and negative regulation of cell death. Galectin-1 is abundant in many organs, especially in the prostate stroma. Galectin-1 was initially reported to kill subsets of B and T lymphocytes. However, recent reports have demonstrated that an androgen-dependent prostate cancer cell line undergoes apoptosis after binding soluble galectin-1, while two androgen-independent prostate cancer cell lines are resistant to galectin-1 induced death. Thus, galectin-1 may be a general death trigger for a variety of cell types. As resistance to apoptosis is a hallmark of many types of cancers, we wish to understand the mechanisms governing susceptibility or resistance of various prostate cancer cell lines to galectin-1 induced apoptosis. Understanding the regulation of prostate cancer cell death will allow the development of novel therapeutic approaches to eliminate tumor cells.

Body

The approved Specific Aims and Statement of Work for this project are below. In our first year of funding, we have made substantial progress in Aims 1 and 2, and Tasks 1 and 2, as described following the Specific Aims and Statement of Work. We have also performed preliminary work essential for the completion of Task 4.

Specific Aims: 1) Characterize features of galectin-1 death in prostate cancer cell lines. 2) Identify the expression pattern of glycosyltransferase genes that regulate galectin-1 cell death in prostate cancer cells, and determine if regulated glycosyltransferase expression controls cell death. 3) Examine whether, as observed in T cells, anti-apoptotic galectin-3 inhibits galectin-1 induced death of prostate cells, and determine if the two galectins compete for common receptors. 4) Characterize the cell surface receptors for gal-1 on prostate cancer cells and compare the pattern of expression of these receptors on galectin-1 sensitive and gal-1 resistant prostate cancer cell lines. Determine the requirement for specific cell surface receptors in galectin-1 mediated death of prostate cells. 5) Investigate the intracellular galectin-1 death pathway, and identify associations between prostate-specific cell surface receptors and common intracellular death pathway components.

Statement of Work

Task 1. To characterize features of galectin-1 death in prostate cancer cell lines (months 1-3).

a. Examine hallmarks of cell death, including membrane lipid assymetry, cell permeability, loss of mitochondrial membrane potential and DNA fragmentation. (months 1-3)

b. Examine calcium flux, caspase activation and cytochrome C release, to identify novel features of the galectin-1 death pathway in prostate cancer cells. (months 1-3)

Task 2. To identify the roles of specific glycosyltransferase genes that regulate galectin-1 cell death in prostate cancer cells (months 1-18).

a. Examine expression pattern of ST6Gal I, ST3Gal I, FucT VII, GnTV and C2GnT glycosyltransferases in galectin-1 sensitive and insensitive LnCaP cell lines (months 1-6).

b. Determine effects of expressing ST3Gal I and FucT VII that mask and C2GnT that create galectin-1 saccharide ligands on prostate cell susceptibility to galectin-1 (months 6-18).

c. Examine prostate cancer biopsies for the pattern of glycosyltransferase expression in primary prostate cancers (months 6-12).

Task 3. To determine whether galectin-3 opposes the effects of galectin-1 in prostate cancer cell lines (months 6-18).

a. Express galectin-3 in a galectin-1 sensitive LnCaP cell line (months 6-9).

b. Determine effect of galectin-3 overexpression on galectin-1 susceptibility (months 9-12).
c. Perform competition experiments with soluble recombinant galectin-1 and galectin-3 (months 12-18).

Task 4. To examine the requirement for specific prostate cell surface glycoprotein receptors for galectin-1 induced death (months 12-36).

a. Following isolation and characterization of receptors, determine receptor expression pattern on galectin-1 resistant cells (months 12-24).
b. Characterize receptor domains that are required for sending the death signal
c. Examine requirements for specific receptors by antibody inhibition, anti-sense transfection or expression of dominant negative receptor constructs in galectin-1 susceptible cells or by expression in galectin-1 resistant cells, (months 24-36).

Task 5. To investigate the intracellular galectin-1 death pathway (months 12-36).

a. Determine effects of protein kinase C and protein phosphatase inhibitors on galectin-1 susceptibility (months 12-18).
b. Identify additional candidate signaling molecules based on identities of cell surface receptors determined in Task 4 (months 18-30).
c. Determine requirement for specific signaling molecules by expression of relevant wildtype or mutant constructs in specific cell lines (months 18-36).
d. Identify association between cell surface receptors and signaling molecules by immunoprecipitation and confocal immunofluorescent microscopy (months 24-36).

Aim 1. Characterize features of galectin-1 death in prostate cancer cell lines.

Prior work had demonstrated that LNCaP prostate cancer cells, that do not express galectin-1, were susceptible to galectin-1 induced cell death (1). We confirmed the susceptibility of LNCaP cells to galectin-1 induced cell death in our laboratory. As shown in Fig. 1, LNCaP cells died in response to galectin-1, as demonstrated by a variety of different methods. First, we determined that LNCaP cells treated with galectin-1 take up propidium iodide, demonstrating increased membrane permeability. Second, we used TUNEL staining to detect fragmented DNA in galectin-1 treated LNCaP cells. In addition, the galectin-1 treated LNCaP cells became annexin V^+, demonstrating membrane lipid asymmetry that is a hallmark of cell death (data not shown). These assays were initially challenging, as in our previous experiments with T cells, we had treated cells in suspension with recombinant human galectin-1 (2). However, because LNCaP cells are adherent, detaching the cells prior to apoptosis assays resulted in very high background cell death. We developed an assay in which adherent cells were treated in plates with 20 μM galectin-1 for up to 6 hr. Cells were detached with only vigorous pipetting immediately prior to flow cytometric apoptosis assays. Importantly, we found that it was imperative to have the tumor cells in suspension for no longer than 1 hr prior to analysis, or there was an unacceptable background level of cell death.

Thus, in fulfilling **Task 1.a**, we have clearly demonstrated by several methods that galectin-1 induces apoptosis of this prostate cancer cell line. This was a critical finding, as the only weakness indicated in the Peer Review Panel Summary Statement was "the need to consider alternatives if the initial LNCaP cell experiments indicate that galectin does not induce apoptosis", and we have now conclusively demonstrated that LNCaP cells are susceptible to galectin-1 induced apoptosis.

Recent work in our lab has demonstrated that, while galectin-1 death of T cells does not appear to require caspase activation, cytochrome C release or calcium flux, we have found rapid re-localization of the mitochondrial endonuclease G to the nucleus in galectin-1 treated cells (Hahn et al, manuscript submitted). Thus, we have added to **Task 1.b** examination of endonuclease G subcellular localization in galectin-1 treated prostate cancer cells, both by immunofluorescence microscopy and subcellular fractionation followed by immunoblotting. These studies are currently in progress.

Aim 2. Identify the expression pattern of glycosyltransferase genes that regulate galectin-1 cell death in prostate cancer cells, and determine if regulated glycosyltransferase expression controls cell death.

While the LNCaP cell line is susceptible to galectin-1 induced apoptosis, the DU145 and PC3 prostate cell lines are not susceptible to galectin-1 death (3, and our data not shown). This finding raises a critical question -- How do prostate cells become resistant to galectin-1 death? Since the prostatic stroma is especially rich in galectin-1 (4), prostate epithelial cells that are susceptible to galectin-1 induced apoptosis could die upon invading through the basement membrane into the stroma. In contrast, prostate epithelial cells that are resistant to galectin-1 induced death could survive invasion into and migration through the stroma. We have previously demonstrated that regulated expression of specific glycosyltransferase enzymes controls susceptibility of T cells to galectin-1 induced death (5,6). This finding suggested that regulated glycosylation would also control prostate cell susceptibility to galectin-1 induced apoptosis.

In the application for the current project, our preliminary data for this Aim demonstrated altered glycosylation of malignant prostate epithelium, compared to normal prostate epithelium, as demonstrated by lectin histochemistry. We had identified target glycosyltransferases to examine in regulating prostate cell susceptibility to galectin-1, based on our prior work with T cell glycosyltransferases; these target enzymes were ST6Gal I, ST3Gal I, FucT VII, GnTV and C2GnT, all of which can regulate T cell susceptibility to galectin-1.

Shortly before we began this work, Vihko and colleagues published an analysis of differential gene expression between androgen-dependent (AD) and androgen-independent (AI) LNCaP subclones (7). Remarkably, this study demonstrated that galectin-1 was highly overexpressed in AI cells compared to AD cells. We have established a collaboration with the Vihko group, and we have examined the gene expression patterns of a variety of glycosyltransferases in the AD and AI LNCaP cells. We have examined differences in expression of glycosyltransferases involved in both N-glycan and O-glycan synthesis. Fig. 2A shows all glycosyltransferase genes on the Affymetrix U95 chip that were differentially expressed between AD and AI LNCaP cells. On the left, AI LNCaP cells showed a modest increase in enzymes that create the core N-glycan structure, as well as the GnT I and GnT II enzymes that initiate formation of complex N-glycans, compered to AD LNCaP cells. On the right, the AI LNCaP cells had a dramatic reduction in expression of the C2GnT enzyme, compared to the AD cells. This down-regulation is even more striking when one considers that two-fold differences in glycosyltransferase expression is sufficient to change the pattern of cell surface glycosylation. As mentioned above, we have shown that the action of the C2GnT, creating core 2 O-glycans, is essential for T cell death, as galectin-1 binds sugar ligands created by the C2GnT (5,6). These results suggested that reduced C2GnT expression and reduced core 2 O-glycan formation would allow AI cells to express galectin-1 and avoid apoptosis. In addition, this observation indicated that a single glycosyltransferase may regulate susceptibility to galectin-1 induced cell death in prostate cancer cells, as we have found in lymphocytes.

As a first step in understanding how glycosylation regulates prostate cell susceptibility to gal-1 induced death, we have treated LNCaP cells with pharmacologic inhibitors of glycosylation and asked what effects these have on gal-1 cell death. As shown in Fig. 2B, O-linked glycans regulate susceptibility of LNCaP to gal-1. Treatment of cells with benzyl-α-GalNAc increased exposure of non-sialylated O-glycans, resulting from inhibition of terminal sialylation; this was confirmed by increased staining with PNA, a lectin that recognizes non-sialylated O-glycans (data not shown). While galectin-1 treatment resulted in substantial death of control-treated cells, benzyl-α-GalNAc treatment further increased LNCaP susceptibility to galectin-1, so that 90% of cells were killed. We also treated cells with deoxymannojirimycin (DMNJ) to inhibit formation of complex N-glycans. DMNJ treatment reduced cell surface complex N-glycans, as demonstrated by reduced staining with the PHA, that detects branched N-glycans (data not shown). However, in contrast to benzyl-α-GalNAc, treatment with DMNJ had no effect on gal-1 death. These results are consistent with the gene expression array

data in Fig. 2A, showing a dramatic reduction in the C2GnT enzyme that regulates O-glycosylation in galectin-1 resistant AI LNCaP cells.

Thus, in our work on **Task 2.a**, we have demonstrated that O-glycans are the primary structures that regulate prostate cancer cell susceptibility to galectin-1, and that N-glycans do not appear to have a significant role in controlling galectin-1 death. In our initial list of enzymes to consider, the ST6Gal I, FucT VII, and GnTV all regulate N-glycosylation; thus, we will not pursue these enzymes at this time. In our current work on **Task 2.b**, we are concentrating on the ST3Gal I and C2GnT enzymes. The ST3 I competes with the C2GnT to prevent core 2 addition to N-glycans. As benzyl-α-GalNAc reduced O-glycan sialylation and increased death, we predict that overexpression of the ST3Gal I will confer resistance to galectin-1 in AD LNCaP cells; we are currently testing this hypothesis by transfecting LNCaP cells with cDNA encoding the ST3Gal I and selecting clones by loss of PNA staining. These cells will be tested for susceptibility to galectin-1, compared to LNCaP cells transfected with vector alone.

We are also focusing on the C2GnT. We are using an siRNA approach to reduce C2GnT expression in AD LNCaP cells, to determine if reduced C2GnT activity is sufficient to render these cells resistant to galectin-1. We are also over-expressing the C2GnT in the AI LNCaP cells to determine if this makes the cells susceptible to galectin-1; this approach, however, may not be as successful as the anti-sense approach, since the AI cells express high levels of endogenous galectin-1 and we may not be able to isolate viable transfectants.

As recommended on page 6 of the Peer Review Panel Summary Statement, we have begun collaborating with Dr. Charles Sawyers' group here at UCLA. Dr. Sawyers' group has developed several prostate cancer cell lines. As shown for the AD and AI LNCaP cells, the LAPC4 AD prostate cell line does not express galectin-1 (Fig. 3). However, as reported by Ellerhorst et al (3), DU145 and PC3 AI cell lines do express galectin-1. Dr. Sawyers' group has also performed gene expression analyses with of AD and AI prostate cell lines derived from LNCaP and LAPC4 cells. We are currently examining this gene expression data to determine if there are additional glycosyltransferase genes with differential expression between AD and AI cells, and to investigate whether these glycosyltransferases participate in the regulation of galectin-1 induced cell death.

Aim 3. Examine whether, as observed in T cells, anti-apoptotic galectin-3 inhibits galectin-1 induced death of prostate cells, and determine if the two galectins compete for common receptors.

We have not yet begun work on the effect of galectin-3 on galectin-1 death of prostate cells, as outlined in **Task 3**. However, we have recently submitted a manuscript demonstrating that, in B cells, galectin-3 expression antagonizes cell death, and that the C-terminal of galectin-3 acts as a dominant negative, inhibiting the anti-apoptotic effect of full-length galectin-3.

Hoyer KK, Pang M, Kuwabara I, Liu F-T, Said JW, **Baum LG**, Teitell MA (2002) An anti-apoptotic role for galectin-3 in diffuse large B cell lymphomas. (manuscript submitted)

As this work was not directly supported by DAMD 17-02-1-0022, I have not listed it in the Reportable Outcomes section. However, this work provides critical information that we will use as we address the tasks in Aim 3.

Aim 4. Characterize the cell surface receptors for gal-1 on prostate cancer cells.

As an initial step in this Aim, it was essential for us to demonstrate that galectin-1 made by prostate cell lines is secreted and bound back to the cell surface. As shown in Fig. 3, the DU145 and PC3 cells synthesize galectin-1, and we and others (3) have found that the DU145 and PC3 cell lines are not susceptible to galectin-1 cell death. As mentioned above, prostate epithelial cells that are resistant to galectin-1 induced death could survive invasion into and migration through the stroma, and could exploit cell surface galectin-1 to bind to counterreceptors in stroma such as laminin (8,9). We performed immunohistochemistry on non-permeabilized

DU145 and PC3 cells, to determine if we could detect galectin-1 on the cell surface (10). As shown in Fig. 4, these cells have abundant cell surface galectin-1, demonstrating that the cells can export cytosolic galectin-1 and bind it back to glycan ligands on the cell surface.

To continue the work in **Task 4**, we are currently preparing galectin-1 affinity columns to isolate galectin-1 counterreceptors from the galectin-1 sensitive LNCaP and galectin-1 resistant PC3 and DU145 cell lines (11). We will compare the patterns of glycoproteins eluted from the affinity column and begin to identify the glycoproteins. We will then investigate which glycoproteins are required to transmit the death signal in LNCaP cells, initially focusing on galectin-1 binding glycoproteins that are present on LNCaP cells but absent on DU145 and PC3 cells.

Aim 5. Investigate the intracellular galectin-1 death pathway.

As described in the Statement of Work, experiments in **Task 5** would begin after the first year of funding, so we have not yet addressed the items described in this task.

Key Research Accomplishments

- Establishment of apoptosis assays that demonstrate susceptibility of prostate cancer cell lines to galectin-1 induced cell death, measuring membrane lipid asymmetry, plasma membrane permeability and nuclear DNA fragmentation.
- Identification of a class of cell surface oligosaccharides, the O-linked glycans, which control prostate cancer cell susceptibility to galectin-1 induced death.
- Characterization of altered glycosyltransferase expression, specifically reduced expression of the C2GnT enzyme, in androgen-independent, galectin-1 resistant cells, compared to androgen-dependent, galectin-1 susceptible cells.
- Identification of a single glycosyltransferase, the C2GnT, that appears to control susceptibility to galectin-1 death in both lymphoid and prostate cancer cells, indicating a common mechanism to regulate of galectin-1 susceptibility in diverse tissue types.
- Demonstration that androgen-dependent prostate cell lines do not express galectin-1, while androgen-independent prostate cell lines synthesize galectin-1 and can export it to the cell surface.
- Establishment of a collaboration with Dr. Pirrko Vihko, Univeristy of Oulu, Finland, to examine differential gene expression that relates to galectin-1 susceptibility between androgen-dependent and androgen-independent LNCaP cells.
- Establishment of a collaboration with Dr. Charles Sawyers, UCLA, to examine differential gene expression that relates to galectin-1 susceptibility in other androgen-dependent and androgen-independent pairs of prostate cancer cell lines, to extend the work we have done on LNCaP cells.

Reportable outcomes

1. We have published a review article on general effects of galectin-1 binding to cells, focusing on the mechanism by which regulated glycosylation controls galectin-1 binding and receptor clustering.

Brewer CF, MC Miceli and **Baum LG** (2002) Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide mediated cellular interactions. *Current Opinion in Structural Biology* 12:616-623

While this paper does not deal solely with prostate cancer cells, the results in Task 2, demonstrating that specific types of glycans regulate prostate cell susceptibility to galectin-1 and altered glycosyltransferase expression in galectin-1 resistant LNCaP cells, was important for formulating the hypotheses presented in this article. Thus, support from DAMD 17-02-1-0022 is acknowledged. A reprint of this article is **Appendix 1**.

2. Dr. Hector Valenzuela, the post-doctoral fellow who has performed the work on this project, will be presenting his findings at the March, 2003, Glycobiology Gordon Conference in Ventura, CA.

3. We have expanded our work in prostate cancer because of our exciting findings. Dr. Karen Pace, a post-doctoral fellow, has applied for a senior post-doctoral fellowship from the NIH SPORE grant on prostate cancer that was recently awarded to UCLA. Dr. Pace will examine the interaction of galectin-1 made by prostate cancer cells with extracellular matrix and the ability of matrix-associated galectin-1 to kill infiltrating T cells, to address the role of galectin-1 in thwarting the immune system's ability to fight prostate cancer. In addition, Dr. Paula Cabrera, who has recently completed her Ph.D. at the University of Buenos Aires in Argentina, will be joining my lab in the spring to examine the role of galectin-1 in prostate cancer cell invasion and metastasis.

4. We have established productive collaborative projects with the groups of Dr. Pirrko Vihko and Dr. Charles Sawyers, as described above.

Conclusions

In the first year of this funded project, we have met several important goals essential for the completion of the work. We have clearly demonstrated that LNCaP cells are susceptible to galectin-1 induced apoptosis by several criteria, a critical step in advancing this research. In further work on Task 1, we will be investigating a new intracellular apoptotic mechanism, nuclear translocation of endonuclease G, as we have recently found that this event occurs during the unique caspase-independent pathway of galectin-1 mediated death of T cells. We have also demonstrated that O-glycans appear to regulate LNCaP cell susceptibility to galectin-1, and specifically that a unique glycosyltransferase, the C2GnT, is down-regulated in galectin-1 resistant LNCaP cells. This is one of the most exciting findings, since it implies that 1) cell surface glycosylation, rather than protein receptors, regulates susceptibility to galectin-1 and 2) there may be a common glycosylation mechanism that regulates galectin-1 susceptibility in epithelial and lymphoid cells. Thus, the protein backbones that bear the essential glycans may be different among different cell types, but common glycosylation patterns allow galectin-1 to send a death signal to the different cell types. As a result of these findings, we are focusing on directly determining the requirement for core 2 O-glycans in triggering prostate cell death by galectin-1, and diminishing our efforts to investigate the contribution on N-glycans. In addition, as we have now observed an inverse correlation between androgen dependence and galectin-1 expression, demonstrating that androgen independent cells are galectin-1 resistant, we will expand our work to include other androgen-dependent and androgen-independent pairs of cell lines provided by our UCLA colleague Dr. Charles Sawyers.

"So what section": Our work indicates that controlling glycosylation may provide a novel therapeutic approach for treating prostate cancer. Altering glycosylation may make prostate cancer cells susceptible to endogenous galectin-1 and to pharmacologic galectin-1, facilitating elimination of tumor cells *in vivo* through apoptosis. As the galectin-1 death pathway appears to be distinct from that mediated by other apoptotic agents, this will allow a synergistic therapeutic approach to kill prostate cancer cells.

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Figure Legends

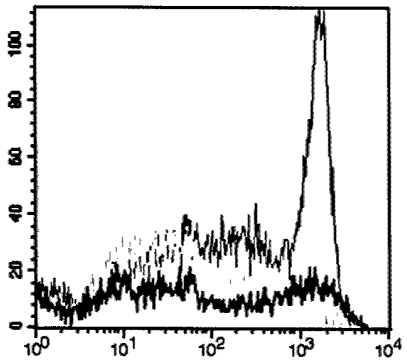
Figure 1. Galectin-1 kills LNCaP cells. Adherent cells were treated with PBS, buffer control or galectin-1 (20 μM) for 6 hours. Cells were detached from the plate with gentle pipetting and apoptosis measured by propidium iodide uptake to measure membrane permeability (left), or TUNEL labeling to detect fragmented DNA (right). For both graphs, the y-axis represents cell number.

Figure 2. Glycosylation regulates prostate cancer cell susceptibility to galectin-1. **A**. Differential expression of glycosyltransferases in AD vs. AI LNCaP subclones. A comparison of gene expression patterns was made between androgen dependent (AD) and androgen independent (AI) subclones of the LNCaP cell line, in collaboration with Dr. Pirkko Vihko. Differential expression of glycosyltransferases involved in the synthesis of N- and O-linked oligosaccharides was observed. Most striking was the 5-fold decrease in C2GnT expression in the AI LNCaP cells compared to the AD LNCaP cells. The individual glycosyltransferases are indicated on the x-axis, and the fold expression relative to the level in AD cells is indicated on the y-axis.
B. O-linked glycans regulate LNCaP susceptibility to galectin-1. Incubation of LNCaP cells with benzyl-α-GalNAc results in increased exposure of O-glycans and increased susceptibility to galectin-1. In contrast, inhibition of complex N-glycan formation with deoxymannojirimycin (DMNJ) had no effect on galectin-1 death. The treatments are indicated on the x-axis, and the percent death is indicated on the y-axis measured by PI uptake. For all experiments, the background level of death seen with buffer alone was subtracted from the percent cell death.

Figure 3. Expression of galectin-1 in prostate cancer cell lines. The AI cell lines DU145 and PC3 demonstrated abundant galectin-1 (14 kD is monomeric form) by immunoblotting, while no galectin-1 expression was detected in the AD cell lines LNCaP and LAPC4.

Figure 4. Soluble galectin-1 is secreted and bound to the surface of AI prostate cells. DU145, PC3 and LNCaP cells were labeled with anti-galectin-1 antiserum. After washing, bound antibody was detected with goat-anti-rabbit antibody conjugated to FITC and cells were analyzed by fluorescent microscopy. Abundant galectin-1 is detected on the surface of the DU145 and PC3 cells. No galectin-1 is detected on the LNCaP cells, consistent with immunoblot analysis in Fig. 3.

Fig. 1 A



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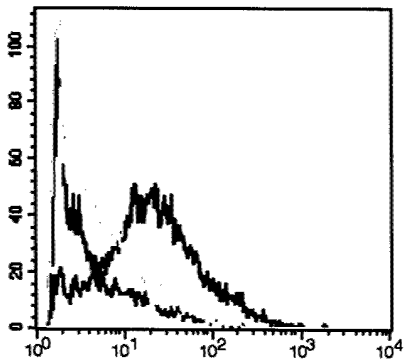
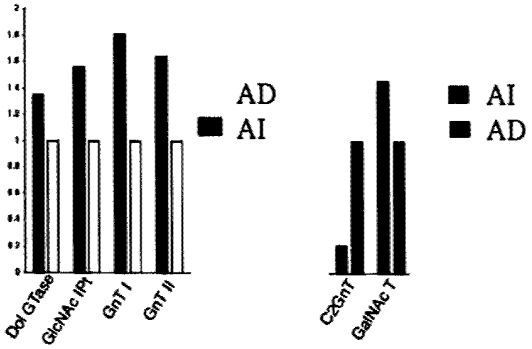


Galectin

Buffer

Media

Fig.2 A



B

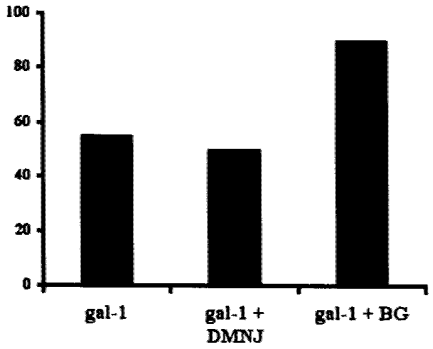


Fig. 3

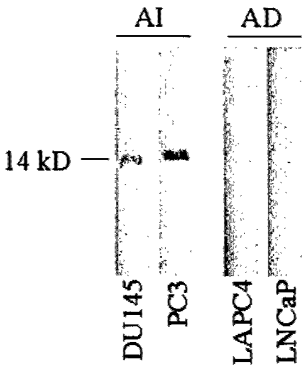
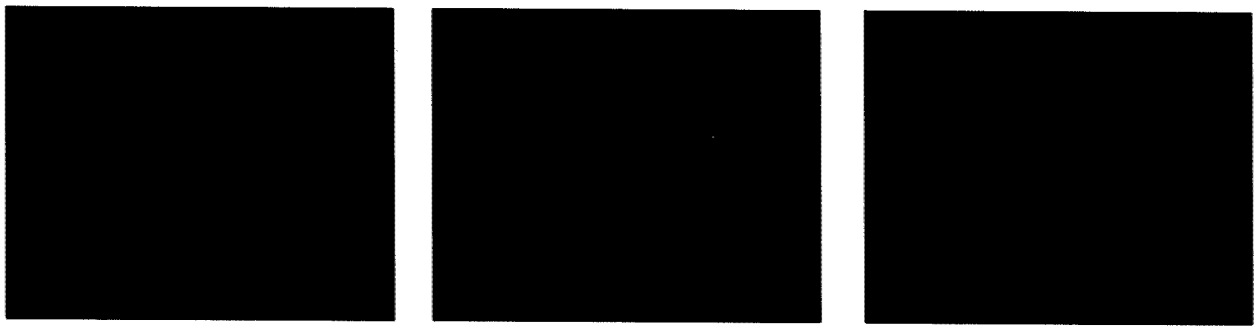


Fig. 4



DU145 PC3 LNCaP

Appendix 1

Clusters, bundles, arrays and lattices: novel mechanisms for lectin–saccharide-mediated cellular interactions

C Fred Brewer*, M Carrie Miceli† and Linda G Baum‡

Multivalent protein–carbohydrate interactions regulate essential cellular events, including cell proliferation, adhesion and death. These multivalent interactions can create homogeneous complexes of lectins, such as the galectins, with their saccharide ligands. Lectin–saccharide complexes can concentrate specific glycoproteins or glycolipids within the lattice, while excluding other cell surface molecules. The formation of lectin–saccharide lattices on the cell surface can thus organize the plasma membrane into specialized domains that perform unique functions.

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Abbreviations

CRD	carbohydrate recognition domain
ECM	extracellular matrix
Gal	galactose
GlcNAc	*N*-acetylglucosamine
MHC	major histocompatibility complex
TCR	T-cell receptor

Introduction

Cells are in constant communication with their surroundings. For normal and pathological processes, including cell proliferation, organ morphogenesis, inflammation, wound healing and tumor metastasis, cells must be able to receive signals from the extracellular milieu and deliver those signals to the inside of the cell. In the past decade, the field of signal transduction has made enormous advances in understanding how 'outside-in' signals are transduced and how those signals are translated inside the cell. However, much of this work has examined protein–protein interactions, that is, the binding of an extracellular protein ligand to a protein receptor on the cell surface, to deliver information from the extracellular milieu to the cell.

Recent work demonstrates that carbohydrate–protein interactions are also critical triggers in cell signaling [1,2•,3,4••,5–7]. These interactions involve the binding of lectins, or carbohydrate recognition proteins, to specific saccharide ligands. Signaling can occur when cell surface lectins transduce a signal after binding saccharide ligands; for example, the B cell lectin CD22 binding to its ligand sialic acid regulates the strength of immunoglobulin signaling [8•,9•]. Signaling can also occur when soluble lectins bind to carbohydrates on cell surface glycoproteins or glycolipids; for example, binding of soluble galectin-1 to its ligand *N*-acetyllactosamine on T cell surface glycoproteins triggers the T cells to die [10–12].

Compared with protein–protein interactions, carbohydrate–protein interactions have several novel features. First, these interactions are regulated at the genetic level by the expression of glycosyltransferase enzymes that create the carbohydrate ligands [4••,11], rather than the direct expression of genes for ligands or receptors. Second, the glycosyltransferases that create a carbohydrate ligand may add that ligand to several different protein backbones on the cell surface. Third, glycoproteins often bear multiple copies of the saccharide ligands that are recognized by lectins [13••,14], either as repeating units on a single oligosaccharide, as seen with mannose residues on yeast glycoproteins [15], or as clustered repeats of the saccharide ligand on the protein backbone, as seen with receptors for the selectins [16]. The lectins that bind the saccharide ligands are often multivalent as well. The multivalent nature of both lectins and their saccharide ligands allows the formation of a lectin–carbohydrate lattice, which acts as a signaling complex at the cell surface.

Lectin–saccharide interactions

Lectin binding to a single saccharide ligand is typically a low-affinity interaction. However, the multivalent nature of lectin–saccharide interactions allows many low-affinity binding events to occur, resulting in high overall avidity [7,17,18]. This multivalency or 'glycoside cluster' effect has been described for several multivalent mammalian lectins. For example, the serum mannose-binding protein preferentially recognizes clustered mannose residues [15] and dimerization of L-selectin increases leukocyte adhesion to clustered saccharide ligands [19,20]. The galectins are a growing family of multivalent lectins [1,7]; several groups have shown that galectins preferentially bind to glycans carrying repeating units of the ligand *N*-acetyllactosamine (Galβ1,4GlcNAc), either as disaccharide units at the termini of tri- or tetra-antennary chains on *N*-glycans, or as repeating units in a poly-*N*-acetyllactosamine chain on *N*- or *O*-glycans [18,21–24].

Multimeric lectins can cross-link multivalent carbohydrate ligands. Given the abundance of saccharides on cell surface glycoproteins and glycolipids, and the ability of glycosyltransferases to add the same glycan structure to

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• D'Alessio KJ, Holmes SD, Abrahamson JA, Erickson-Miller CL, Murdock PR *et al.*: **Identification of SAF-2, a novel siglec expressed on eosinophils, mast cells, and basophils.** *J Allergy Clin Immunol* 2000, **105**:1093-1100.

These two papers [34•,35•] demonstrate the cell-type-restricted pattern of expression of siglecs that is a hallmark of many family members. By using specific monoclonal antibodies, hSiglec-8 is shown to be expressed predominantly on eosinophils in the circulation, but not on neutrophils. Although the cDNA characterised in these studies [34•,35•] does not include ITIM-like motifs, hSiglec-8 has been shown subsequently to also exist in an alternatively spliced form containing ITIM-like motifs [51].

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•• **characterization of a novel mouse Siglec, mSiglec-F: differential evolution of the mouse and human (CD33) Siglec-3-related gene clusters.** *J Biol Chem* 2001, **276**:45128-45136.

The authors characterise a novel mouse CD33-related siglec, mSiglec-F, that is thought to be the orthologue of hSiglec-5, and then characterise the whole murine CD33-related siglec locus using the Celera Genomics database. This analysis reveals striking differences between the murine siglec locus and the human CD33-related siglec locus, and shows that, unlike the other siglecs (CD22, MAG and sialoadhesin), genes encoding the CD33-related siglecs underwent extensive duplications subsequent to mammalian speciation.

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• **characterization of S2V, a novel putative siglec that contains two V set Ig-like domains and recruits protein-tyrosine phosphatases SHPs.** *J Biol Chem* 2001, **276**:23816-23824.

See annotations to [43•,50•].

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• **mouse CD33-related siglec (sialic acid binding immunoglobulin-like lectin) that recruits Src homology 2 (SH2)-domain containing protein tyrosine phosphatases SHP-1 and SHP-2.** *Biochem J* 2001, **353**:483-492.

These studies [41•,43•] identify a novel murine siglec and a novel human siglec-like gene in a yeast two-hybrid screen using SHP-1 as a bait. A detailed characterisation of the interaction between the tyrosine-based motifs of these proteins and tyrosine phosphatases SHP-1 and SHP-2 is undertaken, and the membrane-proximal motif is found to be indispensable for binding in each protein.

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These two groups [41•,50•] identify an unusual human siglec-like molecule, hSiglec-L1 or S2V that contains two tandem V-set domains. The essential arginine that is required for sialic acid binding is replaced by cysteine in the first V-set domain and by glutamine in the second. Although one group [41•] observed sialic-acid-dependent binding in transfected COS cells, the other group did not [50•], but instead showed that mutation of the cysteine to arginine results in robust sialic-acid-dependent binding. Notably, the chimpanzee orthologue has retained the arginine in the first V-set domain and can mediate sialic-acid-dependent binding of human erythrocytes [50•].

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This is the first demonstration that a CD33-related siglec can be phosphorylated on serine residues in the cytoplasmic tail. Evidence is presented that phosphorylation results in altered sialic-acid-binding properties.

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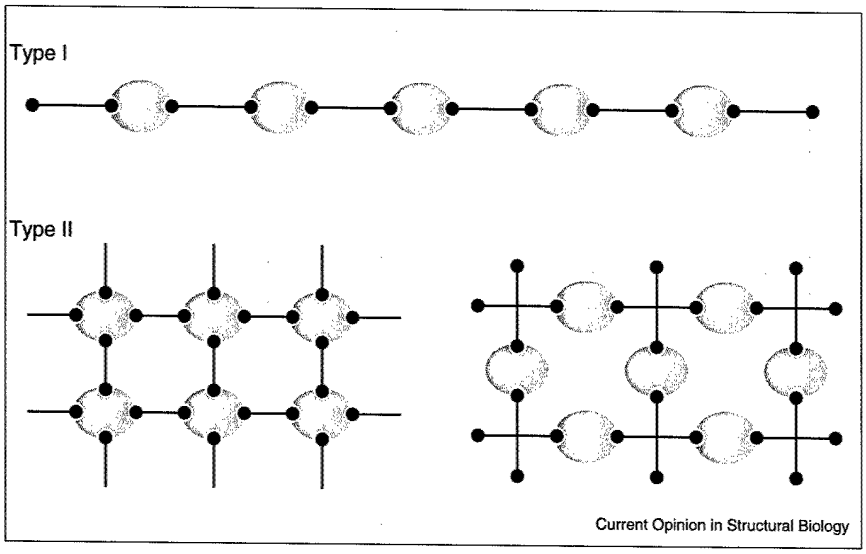
Figure 1



Lectin–saccharide lattices. Type I complexes are composed of bivalent lectins and bivalent carbohydrates. These polymers can be flexible and can accommodate carbohydrate ligands of different lengths. Type II complexes are composed of lectins and carbohydrates, one of which has a valency >2. On the left, a tetravalent lectin is complexed to a bivalent carbohydrate, whereas on the right a bivalent lectin is complexed with a tetravalent carbohydrate. Type II complexes favor inclusion of a single species of carbohydrate ligand, which facilitates the formation of thermodynamically favorable lattices.

different protein or lipid backbones, one might imagine that lectin–saccharide cross-linking could result in one big heterogeneous complex on the cell surface. Surprisingly, several studies with galectins and other lectins have shown that, in contrast to agglutinating a heterogeneous mix of glycoprotein ligands, multivalent lectins selectively cross-link a single species of glycoprotein to form uniform lectin–carbohydrate lattices [3,12,25].

Lectin–carbohydrate interactions can occur in two ways: type I and type II [3] (Figure 1). Type I complexes are formed by bivalent lectins and bivalent ligands, to form linear polymers. These polymers may be polymorphic, because the linear arrangement tolerates flexibility and the ligands can be of different lengths and composition, as long as they are bivalent. By contrast, type II complexes are formed when either the lectin or the ligand has a valency >2. In this case, ordered cross-linked complexes are formed that are homogeneous with respect to ligand, even when several potential ligand species, that is, glycoproteins, are in the mix. The regular spacing in the lattice conferred by the repeating use of a single ligand species is thermodynamically favorable, as shown by studies with a variety of multivalent lectins, including galectins [3,18].

The multivalent galectins

The galectins are a family of lectins defined by a conserved carbohydrate recognition domain (CRD), found in species ranging from fungi to human. Fourteen mammalian galectins have been identified. The structures and biological activities of many of the galectins are the subject of recent excellent reviews [1,7,26] and so will not be examined comprehensively here.

Importantly, all mammalian galectins can act as multivalent lectins. Some galectins, including galectin-1, are synthesized as monomers with a single CRD; these galectins typically dimerize through noncovalent interactions to create functionally bivalent lectins. The tandem-repeat galectins have two CRDs connected by a linker peptide and are thus bivalent, although the two CRDs may be able to recognize slightly different saccharide ligands. Galectin-3 is a chimeric galectin with a C-terminal CRD attached to an N-terminal peptide. However, galectin-3 can also be multimeric, as it can spontaneously form multimers on the cell surface or can be cross-linked by tissue transglutaminase [7].

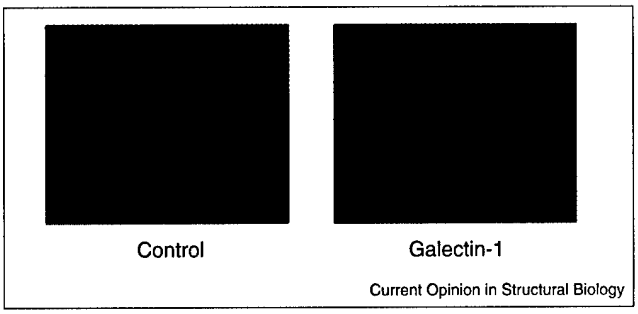
Galectins have a variety of functions in specific tissues and can induce cell proliferation, cell cycle arrest or cell death. Galectins have been implicated in organ morphogenesis, tumor cell metastasis and leukocyte trafficking — all processes that involve adhesion and de-adhesion of different cell types, as well as the recognition of adjacent cells and the extracellular matrix (ECM). Although the *N*-acetyllactosamine ligand recognized by galectins is expressed ubiquitously on *N*- and *O*-linked glycans, galectins can discriminate among different *N*-acetyllactosamine-bearing glycoproteins, which suggests that the preferential recognition of specific *N*-acetyllactosamine-bearing glycoproteins is important for conferring unique galectin functions in specific cell types.

In solution, galectins can discriminate among different multivalent glycoprotein ligands to form homogenous lattices of lectin and glycoprotein [18]. On the cell surface, galectins selectively recognize different glycoprotein ligands [7,25]. This suggests that, on the cell surface, galectins may form homogenous lattices with specific glycoproteins.

Galectin-mediated T cell signaling: lattice control?

In the immune system, programmed cell death is a critical mechanism that avoids the development of autoreactive T cells and limits the extent of an immune response.

Figure 2



Galectin-1-induced clustering of the T cell surface glycoprotein receptor CD45. Before galectin-1 binding, CD45 is distributed uniformly over the surface of the T cell (left). After galectin-1 binding, CD45 is clustered onto membrane 'blebs' that exclude other cell surface glycoproteins (right). Localization of CD45 was detected with a fluorescein-labeled antibody to CD45 and cells were analyzed by confocal microscopy.

Galectin-1, widely expressed in a variety of organs, induces death of developing T cells in the thymus and activated T cells in the periphery [10,27,28]. Several T cell glycoproteins that can bind galectin-1 have been identified, including CD3, CD7, CD43 and CD45 [25,29,30]. This suggested that either galectin-1 bound all of these receptors in a heterogeneous complex on the cell surface or galectin-1 selectively cross-linked specific receptors into a homogeneous signaling lattice, analogous to the lattice formation observed in solution.

To examine these two possibilities, the surface distribution of CD3, CD7, CD43 and CD45 on T cells was examined before and after galectin-1 binding [25]. Pace *et al.* [25] found that galectin-1 selectively forms complexes containing either CD45 and CD3, or CD7 and CD43, resulting in the segregation of CD3/CD45 from CD7/CD43 complexes on the cell surface (Figure 2). Segregation of CD45 from CD7 and CD43 appeared to be essential to initiate cell death; the CD45 cytoplasmic domain has tyrosine phosphatase activity that could antagonize pro-death signals delivered via CD7/CD43, so that the segregation of CD45 may allow the death signal to be delivered unopposed [12]. These studies demonstrated that homotypic lattices of galectin and specific glycoproteins can form on the cell surface and that these lattices regulate the signal delivered by galectin-1 binding.

Galectin-1-mediated lattice formation can also influence signals sent by other receptor–ligand systems. Engagement of the T-cell receptor (TCR) plus a co-stimulatory molecule either by antigen presentation or by cross-linking antibodies causes clustering of TCRs that signal the cell to proliferate and to secrete cytokines [31]. Galectin-1 binding to T cells antagonizes this signal by limiting the clustering of membrane microdomains containing the TCRs after engagement [32] (Figure 3). In this manner, galectin-1 binding to TCRs and/or associated glycoproteins appears to keep the TCRs spaced a critical distance apart on the cell surface, thus acting as a brake on the amplitude, processivity and/or duration of signaling by the TCR.

A similar effect has been ascribed to galectin-3. Dennis and co-workers [4••,5] examined mice lacking the GnT V enzyme, which can create multivalent poly-*N*-acetyllactosamine oligosaccharides that are preferentially recognized by galectins. T cells from the GnT V$^{-/-}$ mice demonstrated enhanced TCR responses, with dramatic TCR clustering after TCR engagement, which suggests that the GnT V enzyme participates in creating the cell surface structures that normally limit the extent of TCR interactions. This effect could be phenocopied by pre-incubating the cells with lactose to dissociate bound galectin-3, suggesting that galectin-3 mediates the GnT-V-dependent negative regulation of T cell signaling. Thus, galectins can act as a brake on TCR signaling, by forming a lattice with specific glycans on TCR glycoproteins to limit the clustering of TCRs after antigen binding.

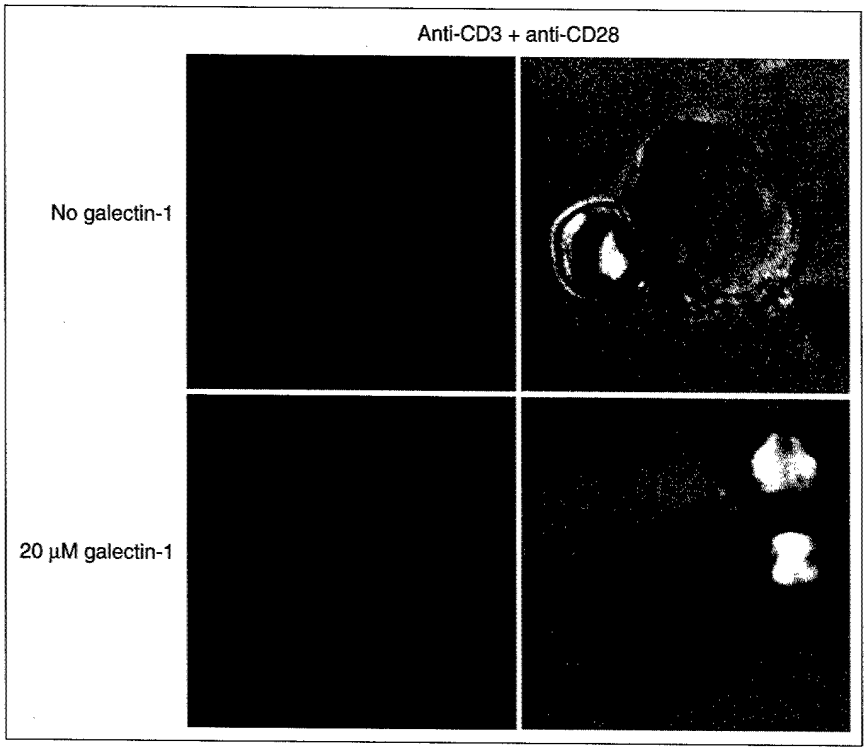
Galectins and cell adhesion: bundles and clusters

As described above, galectins control T cell fate both directly by triggering cell death and indirectly by modulating signals sent through other signaling pathways, such as that of the TCR. A large body of work has examined the roles of galectins in mediating cell adhesion to the ECM, an important process in organogenesis and tumor metastasis (reviewed in [7]). Strikingly, there are again examples showing that galectins directly mediate cell adhesion and also indirectly modulate cell adhesion through other molecules, such as integrins. In both situations, galectins appear to interact with multivalent saccharide ligands, suggesting that formation of the lectin–saccharide lattice is also involved in cell adhesion.

Galectins can bind directly to ECM proteins, such as laminin, that display abundant poly-*N*-acetyllactosamine-containing glycans on the polypeptide backbone [21]. Dimeric galectin-1 directly mediated the binding of smooth muscle cells and melanoma cells to laminin. In contrast, galectin-3 at a low concentration did not mediate melanoma cell–laminin binding; however, after transglutaminase cross-linking, multivalent galectin-3 could mediate melanoma cell–ECM binding, indicating that multivalent lectin binding was critical for adhesion. Galectin-3 also mediates neutrophil adhesion to laminin and, at low concentrations, the adhesion of breast cancer cells to the ECM (reviewed in [7]).

Galectin-1 and galectin-3 can both indirectly influence cell adhesion as well. Galectin-1 antagonizes lymphocyte adhesion to integrins [33], whereas high concentrations of galectin-3 antagonize breast cancer cell adhesion to integrins [34•]. Galectin-3 secreted from epithelial cells has also been found to 'bundle' or polymerize the ECM protein hensin [35]. In the presence of galectin-3, hensin is polymerized and causes terminal differentiation of

Figure 3



Galectin-1 binding antagonizes lipid raft reorganization. In the absence of galectin-1, T cell binding to beads coated with antibodies to CD3 and CD28 results in the relocalization of lipid rafts to the site of engagement (top). In the presence of galectin-1, lipid raft reorganization is prevented and rafts do not cluster at the cell–bead interface (bottom). Lipid rafts were visualized with a green fluorescent protein targeted specifically to lipid rafts by fusion to the N-terminal ten amino acids of the intracellular kinase lck (left) and bright field images of the same cell are shown (right). Reproduced with permission from [32].

epithelial cells, whereas in the absence of galectin-3, hensin remains unpolymerized and has no differentiation effect. Thus, galectin-3 modulates the ECM signal mediated by hensin to direct cell fate.

In addition, galectin-8, a tandem-repeat galectin with two CRDs, has both pro-adhesive and anti-adhesive functions [36]. By binding to cell surface integrins, immobilized galectin-8 can directly mediate the adhesion and spreading of various cell types. But neither truncated galectin-8 with only one CRD nor excess soluble galectin-8 mediates cell adhesion and spreading, which suggests that the physical arrangement of the galectin-8–integrin interactions is important for cell adhesion and spreading, rather than simply the engagement of cell surface integrins.

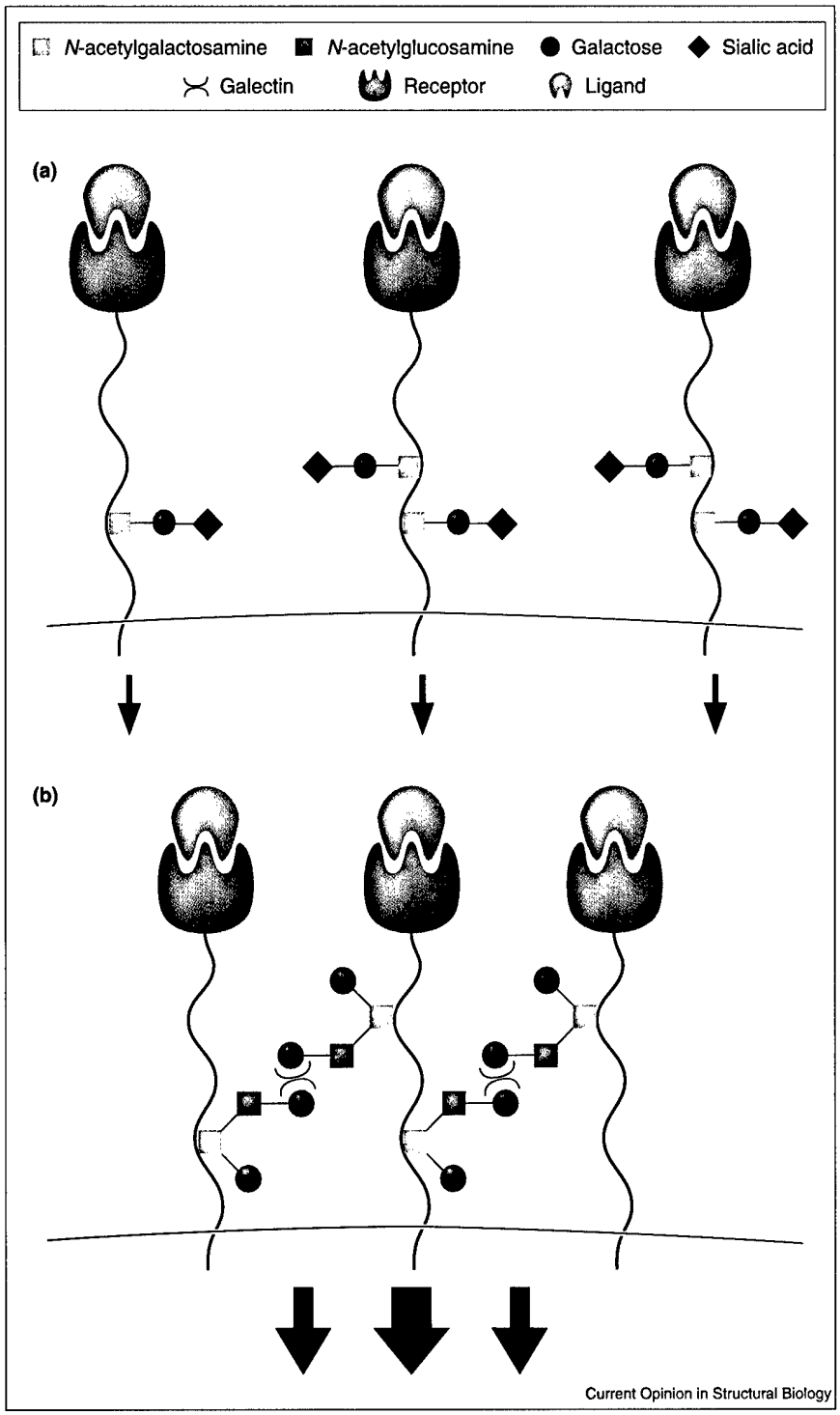
Galectin localization in membrane microdomains: floating in flasks and rafts

The ability of galectins to organize membrane glycoproteins into discrete microdomains on the cell surface suggests that the multivalent lectin–saccharide interactions occur preferentially in these specific domains. A type of lipid microdomain that seems to be essential for assembling signal transduction components at the plasma membrane is the lipid raft, a glycolipid-enriched membrane domain [31,37]. Lipid rafts concentrate specific membrane glycoproteins, glycolipids and cytosolic signaling proteins in order to modulate signals produced when extracellular ligands engage cell surface receptors. For example, lipid rafts containing TCRs coalesce after the TCRs bind antigen on antigen-presenting cells expressing co-stimulatory ligands.

One mechanism by which galectin-1 modulates TCR signaling is by limiting the coalescence of lipid rafts and thus the extent of TCR phosphorylation [32]. Indeed, galectin-1 binding to T cells results in partial but incomplete phosphorylation of the TCR ζ chain. In addition, disrupting lipid raft integrity with the cholesterol-chelating agent methyl-β-cyclodextrin inhibits galectin-1-induced ζ chain partial phosphorylation, implying that lipid rafts are required for galectin-1-mediated effects. Lipid rafts are also rich in the ganglioside GM1 [37] and antibodies to GM1 inhibit galectin-1 binding to neuroblastoma cells, suggesting that cell surface glycoproteins recognized by galectin-1 are concentrated in lipid rafts [38]. Collectively, these data indicate that galectin-1 can selectively associate with specific glycoproteins in lipid raft membrane microdomains.

Intriguing new work also localizes galectin-3 to lipid rafts. As described above, galectin-3 can antagonize the integrin-mediated adhesion of breast cancer cells. Ochieng and co-workers [34•] have found recently that this process occurs in part by the galectin-3-mediated internalization of cell surface integrins into caveolae, flask-shaped invaginations that dip down from lipid rafts to allow clathrin-independent endocytosis. In many cell types, integrins may be excluded from rafts, unless specific cell activation events occur [39,40]. This implies that

Figure 4



Model of galectin-mediated glycoprotein lattices enhancing signaling by an unrelated ligand. **(a)** Individual receptors engage individual ligands and each sends a weak signal. The presence of sialic acid inhibits galectin-mediated cross-linking of the receptors. **(b)** Appropriate glycosylation allows galectin-mediated cross-linking of the receptors. Subsequent engagement by ligands sends signals that are temporally and spatially concentrated to result in an overall increase in signal strength and/or duration.

galectin-3 binding first concentrated and localized cell surface integrins into lipid rafts, prior to caveolae-mediated internalization.

In intestinal epithelial cells, endogenous galectin-4 is defined as a marker of membrane lipid rafts [41]. On these cells, galectin-4 binds to two cell surface brush border enzymes and remains associated with the lipid raft membrane microdomains. Because galectins, including galectin-4, are secreted by a nonclassical secretion pathway and thus cannot associate intracellularly with the brush border enzymes before externalization from the cell [42,43], the association of galectin-4 with specific brush border enzymes in lipid rafts again demonstrates a

preferential recognition of glycoprotein ligands on the cell surface, resulting in the formation of galectin–glycoprotein clusters in specific membrane domains.

Lectin–saccharide interactions: priming the system

Three possible functions for multivalent galectin–saccharide lattices have been described above. First, these interactions can directly signal a specific cell event, such as galectin-1-induced cell death. Second, these interactions can act as a rheostat on other types of signaling events, such as galectin-1 and galectin-3 antagonism of TCR signaling. Third, these interactions can concentrate cell surface glycoproteins into specific membrane domains to allow domain-specific events to occur, such as the galectin-3-mediated endocytosis of integrins via caveolae. A fourth possible function for galectin–saccharide lattice interactions has been suggested by recent work demonstrating the effect of T cell surface glycosylation on T cell development in the thymus [44••,45••]. This work implies that carbohydrate-mediated interactions of T cell surface glycoproteins potentiate the ability of these glycoproteins to bind their cognate protein receptors.

During T cell maturation in the thymus, specific glycosyltransferase enzymes are expressed at precise points during T cell development. Recently, Moody *et al.* [44••] and Daniels *et al.* [45••] have found that expression of a specific sialyltransferase, ST3Gal I, increases cell surface sialylation and reduces the ability of the CD8 antigen carried on developing T cells to bind major histocompatibility complex (MHC) class I molecules. The binding of the MHC class I protein complex to CD8 is a well-characterized protein–protein interaction and MHC recognition is an important step in proper T cell maturation that allows T cells in the periphery to recognize foreign antigens. Expression of ST3Gal I can also reduce galectin-1 binding to T cell surface glycoproteins, by competing for substrate with the branching enzyme that catalyzes the extension of poly-*N*-acetyllactosamine chains that are preferentially recognized by galectin-1 [11]. This suggests that, in the absence of sialylation, the cross-linking and clustering of cell surface glycoproteins mediated by galectin-1 may enhance the binding of MHC class I molecules to CD8 antigens on developing T cells [46]. There may be other systems in which galectin-mediated cross-linking and clustering of cell surface glycoproteins 'organize' receptors on the cell surface to enhance ligand binding and to increase the amplitude of subsequent intracellular signals (Figure 4).

Conclusions

Traditionally, the transmission of outside-in signals to a cell has been envisaged as a bimolecular interaction in which a protein ligand binds a protein receptor that sends a signal into the cell. It is now becoming clear that, for many systems, the clustering of protein receptors and ligands into a 'synapse' is required for optimal signaling [31], although the forces driving that clustering are still poorly understood [47,48]. However, the model of multivalent protein–carbohydrate interactions discussed here involves the assembly of ordered arrays of lectins and saccharides into large, discrete domains on the cell surface. We therefore propose that the assembly of these arrays, a thermodynamically favorable process, drives subsequent cellular events such as proliferation, adhesion or death. Physiologically, a cell may be covered with endogenous lectins binding to saccharide ligands to create local associations of specific glycoproteins and glycolipids in specific membrane domains. These associations, which are made up of several low-affinity interactions, may be potentiated or antagonized by the engagement of additional signaling molecules. The variables that govern assembly of these lattices might include branching of saccharide ligands [4••,11], presentation of the ligands along protein or lipid backbones [16–23], and the spacing distance of the binding domains of multivalent lectins [49]. Recent work has shown that this type of lectin–saccharide clustering occurs during the interaction of myelin-associated glycoprotein, a lectin, with the glycolipid receptors, GD1a and GT1b, on nerve cells — a process that triggers neurite outgrowth and that may be modulated by other signaling pathways [50]. Given that virtually every protein on the surface of a cell is glycosylated, lectin–saccharide lattices may be ubiquitous participants in all types of cellular communication.

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