Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis

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We dedicate this work to the memory of Rina Zakut

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SUMMARY

The interaction of cells with the extracellular matrix regulates cell adhesion, motility, growth, survival and differentiation through integrin-mediated signal transduction. Here we demonstrate that galectin-8, a secreted mammalian β-galactoside binding protein, inhibits adhesion of human carcinoma (1299) cells to plates coated with integrin ligands, and induces cell apoptosis. Pretreatment of the cells with Mn2+, which increases the affinity of integrins for their ligands, abolished the inhibitory effects of galectin-8. The inhibitory effects of galectin-8 were specific and were not mimicked by plant lectins or other galectins (galectin-1 and galectin-3). In accordance with its anti-adhesive effects, transfection of galectin-8 cDNA into 1299 cells significantly reduced (by 75%) colony formation, when compared to the number of colonies formed by cells transfected with an empty vector. Affinity chromatography over immobilized galectin-8 indicated that few membrane proteins interacted with galectin-8 in a sugar-dependent manner. Microsequencing and western immunoblotting revealed that αβ1 integrin derived from 1299 as well as other cells (e.g. HeLa and human endothelial cells) is a major galectin-8 binding-protein. Furthermore, immunoprecipitation and immunohistochemical studies suggested that endogenous galectin-8, secreted from 1299 cells, forms complexes with αβ1 integrins expressed on the surface of 1299 cells. Galectin-8 also interacts with other members of the integrin family, like αβ1 integrins. In contrast, galectin-8 only minimally interacts with α or β integrins. We propose that galectin-8 is an integrin binding-protein that interacts to a different extent with several, but not all members of the integrin family. Binding of galectin-8 modulates integrin interactions with the extracellular matrix and thus regulates cell adhesion and cell survival.

Key words: Galectin, Adhesion, Integrin, Apoptosis

INTRODUCTION

Cell-matrix interactions depend to a large extent upon the engagement of specific ligands by members of the integrin family of cell-adhesion receptors (Clark and Brugge, 1995; Hynes, 1999; Schwartz et al., 1995). Integrins mediate cell adhesion, migration and invasion, and were implicated in the regulation of many cellular functions including embryonic development, tumor cell growth, programmed cell death, leukocyte homing, bone resorption, clot retraction, and the response of cells to mechanical stress (Clark and Brugge, 1995). Integrins also have a multitude of intracellular effects, both on cytoskeletal organization and as triggers of an intricate network of signalling pathways (Giancotti and Ruoslahti, 1999; Hynes, 1992; Lewis and Schwartz, 1995; Miyamoto et al., 1995a,b).

Integrins are a family of α and β subunits (Hynes, 1992), where each β subunit complexes with a distinct set of α subunits to create heterodimers with characteristic binding specificities. The topographies of α and β subunits are similar. The majority of each is extracellular, and amino-terminal regions, mainly of the α subunits, comprise the ligand binding pocket. Ligand binding, cell adhesion and other properties of integrins require divalent cations (Stuiver and O’Toole, 1995). Most integrins bind ligands that are components of extracellular matrix (ECM) (e.g. fibronectin, collagen and vitronectin), and both ligand occupancy and integrin clustering are critical for triggering intracellular integrin-mediated responses.

Integrins do not function merely as adhesion receptors. Rather, they play a larger role in bidirectional signaling with the extracellular environment (Hynes, 1992). Integrins are characterized by their ability to dynamically regulate their ligand binding affinity, a process termed ‘activation’. Integrins can be activated by manipulating extracellular ions, and by treating cells with certain monoclonal anti-integrin antibodies. This type of integrin modulation is caused by a direct conformational change of the integrin molecule (Ginsberg et al., 1992). Integrin activation might also take place as a result of intracellular signals or ‘inside out signalling’ (cf. Williams et al., 1994, for review). This process involves both α and β subunit cytoplasmic domains and it depends upon the cell type (Hughes et al., 1996; O’Toole et al., 1994). Several signalling molecules were implicated in modulating integrin activation;
these include heterotrimeric G-proteins, lipids and phospholipid metabolites, Ser/Thr and Tyr kinases, as well as small GTP-binding proteins (cf. Zhang et al., 1996, and references therein). Nonetheless, the intracellular signalling pathways and proteins involved in modulating integrin-mediated cell adhesion remain largely unknown.

Cytoplasmic signalling pathways that suppress integrin activation exist as well. PDGF inhibits platelet aggregation, suggesting that activation of a receptor tyrosine kinase impairs the function of integrin \( \alpha_{IIb}\beta_3 \) (Vassbotn et al., 1994). Phosphorylation of a conserved Ser residue in the \( \beta_1 \) cytoplasmic domain may account for the loss of integrin function in mitotic cells (Hynes, 1992), and a \( \beta_1 \)-integrin-linked Ser/Thr kinase (p59\( \text{ILK} \)) that phosphorylates the \( \beta_1 \)-integrin cytoplasmic domain inhibits adhesion to integrin substrates (Hannigan et al., 1996). Similarly, a Ras/Raf-initiated MAP kinase pathway was shown to suppress integrin activation in a transcription-independent manner (Hughes et al., 1997). Signalling events initiated by the occupancy of one integrin can suppress functions associated with other integrins, a process known as integrin crosstalk (Blaystone et al., 1994), or as trans-dominant inhibition (Diaz-Gonzalez et al., 1996; Huhtala et al., 1995).

Cell adhesion also depends upon carbohydrate-protein interactions, mediated by lectins of different families (Sharon and Lis, 1995). Selectins, a family of cell surface glycoproteins, mediate lymphocyte homing to peripheral lymph nodes and are involved in both neutrophil and lymphocyte adhesion to activated endothelial cells (Springer, 1995). Similarly, galectins, animal lectins which specifically bind \( \beta \)-galactoside residues (Barondes et al., 1994a), were implicated as modulators of cell adhesion. While lacking a signal peptide and found mainly in the cytosol, galectins are externalized by an atypical secretory mechanism (Hughes, 1999) to mediate cell growth, cell transformation, embryogenesis (reviewed in Barondes et al., 1994b) and apoptosis (Perillo et al., 1995). In accordance with their proposed functions, galectins enhance (Kuwabara and Liu, 1996; Mahanthappa et al., 1994; Zhou and Cummings, 1993) or inhibit (Cooper et al., 1991; Sato and Hughes, 1992) cell-matrix interactions. Though little is known about the exact role of galectin in inhibiting cell adhesion, current models suggest that binding of galectins to matrix proteins elicits an inhibitory effect due to steric hindrance of cell-matrix interactions (Cooper et al., 1991; Sato and Hughes, 1992).

Galectin-8 (Hadari et al., 1997a, 1995), a member of the galectin family, is a 34 kDa secreted protein, made of two homologous (38% identity) carbohydrate-recognition domains (CRDs) linked by a short (approx. 26 amino acids) linking peptide. Here we show that galectin-8 is an integrin binding-protein that interacts with several, but not all, members of the integrin family. A conformational change induced upon binding of galectin-8 to integrins presumably accounts for the inhibitory effects of galectin-8 on cell adhesion and its ability to induce apoptosis. These events do not involve a steric hindrance of cell-matrix interaction, but rather result from anti-adhesive effects of galectin-8. Thus, galectin-8 represents a novel class of cell adhesion modulators that bind sugar moieties of integrins to regulate integrin interactions with the extracellular matrix and the signals generated thereof.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from Fermentas, radiolabeled nucleotides were from Amersham (Buckinghamshire, UK), and all other reagents were from Sigma unless stated otherwise. Galectin-1 and galectin-3 were a generous gift of Dr Hans-Joachim Gabius (München, Germany).

Cell cultures

1299 cells (derived from human non-small cell lung carcinoma; Mitsudomi et al., 1992) and bovine aorta endothelial (BAE) cells were grown in RPMI medium containing 10% fetal calf serum (FCS). Human HeLa cells were grown in MEM medium containing 10% FCS; Madin-Darby canine kidney (MDCK), L-8 muscle cells, human keratinocytes (Hakat cells) and human hepatoma Hep-G2 cells were grown in DMEM medium containing 10% FCS. Granulosa H023 cells and human endothelial cells were grown in DMEM/F12 medium containing 10% FCS.

Antibodies

Antisera against galectin-8 was raised in rabbits by injection of rGalectin-8 protein, purified over lactosyl-Sepharose (Hadari et al., 1995). The antibodies were affinity-purified over a column of Protein-A coupled to Sepharose, and were eluted with 0.1 M glycine, pH 2.7 (IgG fraction). Rabbit anti-human integrin \( \alpha_3 \) polyclonal antiserum (AB1920) directed towards the intracellular domain of \( \alpha_3 \) was purchased from Chemicon International, CA, USA, and was used for immunoprecipitation; mouse anti-human \( \alpha_3 \) monoclonal antibodies, directed towards the extracellular domain of \( \alpha_3 \) integrin, were purchased from Serotec, UK, and were used for immunofluorescence and immunohistochemistry according to the manufacturer’s instructions. Western immunoblotting was carried out using \( \alpha_3 \) or \( \beta_1 \) antibodies that were kindly provided by Dr K. Yamada (NIH, Bethesda, MD, USA). The following IgG-purified murine monoclonal antibodies against human antigens were purchased from Serotec: anti-\( \alpha_3 \) (VLA-3, CD49c), anti-\( \alpha_4 \) (VLA-4, CD49d), anti-\( \alpha_5 \) (VLA-6, CD49f) and anti-\( \beta_3 \) (CD-29, MCA1189). Fluorescein (FITC)-conjugated affinity-purified F(ab’2) fragment of goat anti-mouse IgG (H+L) was purchased from Jackson Immunoresearch Laboratories, Inc.

Generation of galectin-8/docDNA-3 expression plasmids

An EcoRI restriction fragment encoding the full-length cDNA of rat galectin-8 (nucleotides 1-1241) in the Bluescript expression plasmid (Hadari et al., 1995) was excised with EcoRI and ligated, both in the sense and anti-sense orientations, into the pcDNA-3 (Invitrogen) expression plasmid.

Purification of rGalectin-8

Bacterially expressed recombinant galectin-8 (rGalectin-8), eluted from a column of lactosyl-Sepharose (Hadari et al., 1995), was further purified over an FPLC column. Samples (0.5 ml) of rGalectin-8 (approx. 2 mg/ml) were applied to Superdex-200 (Pharmacia) column, equilibrated with phosphate-buffered saline (PBS), and 0.5 ml fractions were collected at a rate of 0.5 ml/minute. rGalectin-8-containing fractions were pooled, and maintained at 4°C. This preparation of galectin-8 was used throughout these studies.

Agglutination assay

The biological activity of the lectins under study was assayed by measuring their ability to agglutinate formaldehyde-fixed, trypsin-treated rabbit erythrocytes. Rabbit erythrocytes were trypsin-treated according to Lis and Sharon (1972). Hemagglutinating activity and inhibition of hemagglutination by different sugars were assayed by serial dilution in microtiter U-shaped plates as described (Lis and Sharon, 1972). The minimal inhibitory concentration of different
sugars was measured by mixing serially diluted sugars with galectin-8 (0.5 μM) in a final volume of 50 μl, 20 minutes before adding 50 μl of a 4% suspension of packed rabbit erythrocytes in PBS for 30 minutes at 22°C.

**Plating of 1299 cells**
Confluent 1299 (human lung carcinoma) cells were suspended by incubation for 15 minutes at 37°C with PBS in the presence of 5 mM EDTA. Cells were washed, resuspended in RPMI medium containing 10% FCS, and seeded in 24-well Costar plates (3x10^5 cells/well).

**Secretion of galectin-8**
1299 cells, grown to confluence in 10 cm plates, were incubated for 24 or 48 hours in serum-free medium (10 ml/plate). At the end of incubation the medium was collected and centrifuged (5 minutes, 300 g, 22°C). Deoxycholate (DOC; 0.5 mg/ml) and trichloroacetic acid (TCA; 10%) were added to the supernatant. The precipitated proteins were collected by centrifugation at 4°C for 10 minutes, 10,000 g. The pellets were washed twice with 20 ml ethanol/ether (1:1 v/v), and resuspended in 200 μl of Laemmli’s sample buffer (Laemmli, 1970). The precipitated proteins were then resolved by 12% SDS-PAGE and western immunoblotted with anti-galectin-8 antibodies.

**Trypsinization of galectin-8**
1299 cells, grown to confluence on 15 cm plates, were resuspended by incubation with 10 ml of PBS in the presence of 5 mM EDTA at 15 minutes at 37°C. The resuspended cells were washed four times with PBS, and were finally resuspended in 1 ml PBS to yield 4x10^6 cells/ml. Samples (100 μl) of this suspension were incubated in the absence or presence of 0.05% trypsin (250 U/ml) for 30 minutes at 37°C. Following incubation, cells were centrifuged for 5 minutes at 1500 g at 24°C, and were extracted in buffer A (50 mM Heps, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, pH 7.5).

**Overexpression of galectin-8**
6x10^6 1299 cells were transfected by electroporation (960 μF, 250 V) with 15 μg DNA of pcDNA-3 vector alone, or with a vector containing rat galectin-8 cDNA in the sense or anti-sense orientation. Following transfection, cells were plated in non-selective medium (RPMI containing 10% FCS), and 48 hours thereafter, 0.5 mg/ml of G-418 was added for selection of stable colonies. The medium (including G-418) was replaced every 3 days, and at day 21 colonies were stained (Hadari et al., 1997b).

**Binding of 35S-labeled proteins to GST-galectin-8**
1299 cells were grown to confluence on 10 cm plates and were incubated for 16 hours in the presence of 1 μg **(35S)-Met/leucine** and **(35S)-Cysteine; Pro-Mix, Amersham**. Labeled cells were extracted in 400 μl of buffer B (25 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1% NP-40, pH 7.5) and the homogenates were centrifuged for 15 minutes at 4°C to 12,000 g. Supernatants were collected and considered as total cell extract. Cell extracts (300 μl, 1.8 mg) were incubated for 2 hours at 4°C with immobilized glutathione s-transferase (GST) or GST-Galectin-8, in the presence or absence of 10 mM thiodigalactoside (TDG), as indicated. At the end of incubation the beads were washed three times with buffer B, twice with buffer C (25 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, pH 7.5) and boiled in Laemmli sample buffer. The proteins were resolved by means of 7.5% SDS-PAGE and subjected to autoradiography.

**Immunoprecipitation**
1299 cells were extracted in buffer D (25 mM Tris/Cl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, pH 7.5). Cell extracts (1 mg) were immunoprecipitated with the indicated antibodies (16 hours at 4°C). The immunoprecipitated extracts were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and western immunoblotted with anti-galectin-8 antibodies.

**Amino-terminal sequencing**
Two 15 cm plates of 1299 cells were extracted and incubated for 2 hours at 4°C with immobilized GST-galectin-8 as described above. Bound proteins were resolved by means of 7.5% SDS-PAGE, and blotted to PVDF membranes. Following staining, the 125 kDa band was excised and subjected to amino-terminal sequencing as described (Hadari et al., 1997b).

**Immunofluorescence labeling**
1299 cells, grown to confluence on slides, were washed three times with PBS and fixed with 3% paraformaldehyde. Immunolabeling with galectin-8 antibodies (80 μg/ml) was carried out as previously described (Hadari et al., 1993).

**Immunohistochemistry**
1299 cells were incubated for 1 hour in serum-free medium with or without 1 μM rGalectin-8. Cells were washed three times in serum-free medium, fixed with 3% paraformaldehyde, and blocked for 1 hour at 24°C with Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% BSA. Cells were washed three times with TBST buffer and further incubated for 16 hours at 4°C in TBST buffer containing 0.3% Triton X-100 and mouse antibodies directed towards cell-surface epitopes, as indicated. Cells were washed and further incubated for 1 hour at 24°C with biotin-conjugated goat anti-mouse antibodies in TBST buffer supplemented with 5% BSA. Following additional washes in TBST buffer cells were incubated for 1 hour at 24°C with horseradish peroxidase (HRP)-streptavidin, cells were washed and bound antibodies were detected using the color AEC substrate kit (Zymed).

**Flow cytometry**
The level of expression of cell-surface integrins was determined by flow cytometry. Different cell lines, as indicated, were grown to confluence on 6-well Costar plates. Cells were suspended in PBS-EDTA (5 mM) buffer and washed in PBS. Cells (0.5-1x10^6 per sample) were stained with the indicated mouse anti-integrin antibodies (1 μg) in PBS containing 10% FCS and 0.02% (w/v) NaN₃ for 1 hour at 4°C. Following incubation cells were washed and stained with goat anti-mouse FITC-conjugated antibody for 1 hour at 4°C and were washed again. Data were acquired using FACSCAN flow cytometer (Becto Dickinson).

**Apoptosis**
1299 cells were grown to confluence on 6-well Costar plates. Adherent cells were treated as indicated, resuspended in 1 ml of PBS containing 5 mM EDTA, and collected by centrifugation at 1500 rpm for 5 minutes at 22°C. Cells were fixed in methanol at ~20°C for 30 minutes, washed in PBS and resuspended in 0.5 ml of PBS containing 25 μg/ml propidium iodide and RNase A (50 μg/ml). Fluorescence of propidium-iodide-stained cells was measured with a FACSCAN flow cytometer (Becto Dickinson). Equal numbers of events from different treatments were recorded separately. To detect nuclear fragmentation, 1299 cells, grown on slides, were treated under different conditions, washed with PBS, and fixed with methanol (~20°C, 10 minutes) and acetone (~-20°C, 10 minutes). Slides were washed with PBS, incubated with DAPI (0.5 μg/ml PBS) at 22°C for 30 minutes, washed again and viewed under the fluorescence microscope.

**RESULTS**
Recombinant galectin-8 attenuates cell adhesion
Incubation of bacterially expressed recombinant galectin-8
(rGalectin-8) (0.5 μM) with a suspension of 1299 (human lung carcinoma) cells, prior to their seeding on tissue culture plates, resulted in a marked attenuation in the rate of cell adhesion (Fig. 1A). While a significant fraction of the control cells was already adherent after 2 hours, the galectin-8-treated cells failed to spread, and most of them remained floating in the medium. Inhibition of cell adhesion by galectin-8 was dose-dependent, with a half-maximal inhibitory effect achieved at a concentration of 0.1 μM, and a maximal inhibitory effect at 1 μM (Fig. 1B). The inhibitory effects were abolished either by heat inactivation of the galectin protein (not shown), or upon inclusion of 10 mM thiodigalactoside (TDG), which blocks lectin-carbohydrate interactions (Fig. 1C). Similarly, adhesion of 1299 cells was markedly inhibited (80%) when they were seeded on plates precoated with galectin-8 (Fig. 1D). These results indicate that a functional galectin-8 inhibits cell adhesion in a process that involves protein-sugar interactions. Inhibition of cell adhesion, induced by galectin-8, was not due to some form of general toxicity since incubation of the suspended 1299 cells with galectin-8 did not affect cell viability, as assayed by Trypan Blue exclusion (not shown). The inhibitory effects of galectin-8 were not restricted to 1299 cells. Galectin-8 effectively inhibited (albeit with a different potency) adhesion of other cell lines including human keratinocytes (Hakat cells), human HeLa cells and human hepatoma Hep-G2 cells (Table 1) as well as CHO fibroblasts and rat pheochromocytoma (PC-12 cells) (not shown). Maximal doses of galectin-8 (2 μM) induced 50-80% inhibition of the above cell lines.

The inhibitory effects of galectin-8 on cell adhesion are specific

To assess the specificity of the inhibitory effects exerted by galectin-8, we compared it to a panel of other galectins (galectins-1 and galectin-3) and plant lectins (soybean toadstool lectin, wheat germ agglutinin (WGA), Concanavalin A (Con A) or ECorL) promoted the ability of 1299 cells to adhere to these plates, while adhesion of cells to plates precoated with galectin-8 was severely inhibited (Fig. 2B). These findings indicated that the anti-adhesive effects of galectin-8 were due to specific interactions of galectin-8 with cellular proteins, and could not be attributed to some trivial cross-linking of a sufficient number of cell surface proteins.

Sugar-binding specificity of galectin-8

Failure of other galectins and plant lectins to inhibit cell adhesion suggested that galectin-8 might have a unique sugar-binding specificity. To address this possibility we studied the ability of different sugars to inhibit the hemagglutinating activity of galectin-8. As shown in Table 2, agglutination of red-blood cells, induced by galectin-8, was effectively inhibited by polylactosamine, lactose and other sugars containing galactose moieties, but not by glucose, mannose or fucose. Overall, the sugar-binding specificity of galectin-8 conformed to the expected specificity of galectins although it showed some unique features. For example, galectin-1 interacted with TDG or lactosamine with a similar affinity that was four to fivefold higher than its affinity for lactose (Leffler, 1986). In contrast, galectin-8 bound lactosamine, lactose and other sugars containing galactose moieties, but not by glucose, mannose or fucose. Overall, the sugar-binding specificity of galectin-8 is in line with previous findings (Carter et al., 1981; Oppenheimer-Marks and Grinnel, 1981), coating of tissue culture plates with wheat germ agglutinin (WGA), Concanavalin A (Con A) or ECorL promoted the ability of 1299 cells to adhere to these plates, while adhesion of cells to plates precoated with galectin-8 was severely inhibited (Fig. 2B). These findings indicated that the anti-adhesive effects of galectin-8 were due to specific interactions of galectin-8 with cellular proteins, and could not be attributed to some trivial cross-linking of a sufficient number of cell surface proteins.

### Table 1. Galectin-8 inhibits adhesion of cell lines expressing different repertoires of integrins

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (μM)</th>
<th>Integrins (relative abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hep-G2</td>
<td>1</td>
<td>α2(2.0)·α3(1.0)·α4(0.7)·αα(0.4)</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.5</td>
<td>α2(1.0)·αβ(0.2)</td>
</tr>
<tr>
<td>HAKAT</td>
<td>0.75</td>
<td>α2(1.5)·αβ(0.7)·αα(1.0)</td>
</tr>
<tr>
<td>1299</td>
<td>0.1</td>
<td>α3(1.0)·αα(0.6)·αβ(0.2)</td>
</tr>
</tbody>
</table>

A suspension (3×10³/ml) of the indicated cells was made in RPMI medium containing 10% fetal calf serum. Cells were seeded in 24-well Costar plates, and were decorated with the indicated mouse anti-integrin antibodies (1 μg) for 1 hour at 4°C. Following incubation cells were washed and stained with goat anti-mouse IgG-FITC-conjugated antibody. Data were acquired by flow cytometry as described in Materials and Methods. The relative abundance of integrin α3 was assigned arbitrarily to 1.

### Table 2. Sugar-binding specificity of galectin-8

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Minimal inhibitory concentration (mM)</th>
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<tbody>
<tr>
<td>D-Fuc realloc</td>
<td>&gt;500*</td>
</tr>
<tr>
<td>Glc</td>
<td>&gt;200*</td>
</tr>
<tr>
<td>Man</td>
<td>&gt;200*</td>
</tr>
<tr>
<td>Meα-Gal</td>
<td>&gt;200*</td>
</tr>
<tr>
<td>Meβ-Gal</td>
<td>&gt;200*</td>
</tr>
<tr>
<td>GalNAc</td>
<td>62</td>
</tr>
<tr>
<td>Galβ1→4Glc (lactose)</td>
<td>6</td>
</tr>
<tr>
<td>TDG (Thio-D-galactoside, D-Galactopyranosyl)</td>
<td>7.8</td>
</tr>
<tr>
<td>β-D-thio-galactopyranoside</td>
<td></td>
</tr>
<tr>
<td>4-Npt-Gal (p-nitrophenylox-galactoside)</td>
<td>&gt;50*</td>
</tr>
<tr>
<td>LacNAc (N-acetyl lactosamine)</td>
<td>12</td>
</tr>
<tr>
<td>LNt (lacto-N-neotetraose, β-D-Gal-[1-4]-β-D-GlcNAc-[1-3]-β-D-Gal-[1-4]-D-Glc)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Highest concentration tested.

‡The carbohydrate nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature. Gal, Glc and GalNAc are in the D-configuration, Fuc is in the L-configuration, and all sugars are in the Pyranose form, unless indicated otherwise.

Inhibition of hemagglutination activity of galectin-8 by different sugars was assayed as described in Materials and Methods. The minimal inhibitory concentration of the indicated sugars was measured by mixing serially diluted sugars with galectin-8 (0.5 μM) in a final volume of 50 μl. 20 minutes thereafter a 50 μl sample of a 4% suspension of packed rabbit erythrocytes in PBS was added and incubation was continued for 30 minutes at 22°C.
Fig. 1. Recombinant galectin-8 inhibits cell adhesion. (A) A suspension (3x10^5/ml) of 1299 cells was made in RPMI medium containing 10% fetal calf serum. Cells were seeded in 24-well Costar plates in the absence (■) or presence (●) of 0.5 µM galectin-8. At the indicated times, non-adherent cells were washed, and adherent cells were collected and counted. (B) 1299 cells were plated in the presence of increasing concentrations of galectin-8, and the number of adherent cells was determined following a 2 hour incubation period. (C) 24-well Costar plates were coated with 5 µg of fibronectin (FN), fibronectin-120 (FN-120), laminin or polylysine (PolyLys) for 16 hours at 4°C. The wells were washed with PBS, and blocked (1 hour at 22°C) with 0.1% BSA in PBS. 1299 cells were then added in the absence or in the presence of 0.5 µM galectin-8. When indicated, 1 mM Mn^2+ or 10 mM TDG were added together with the lectin. Following a 2 hour incubation cells were washed and the number of adherent cells was counted. Values are means ± s.d. of triplicate measurements of a representative experiment. (D) Plates remained uncoated or were coated with galectin-8 (25 µg/ml) in the absence or presence of 10 mM TDG, for 2 hours at 37°C. Following the first coating with galectin-8, some plates, as indicated, were further coated (1 hour at 37°C) with 300 µl of galectin-8 antibodies (10 µg/ml). Antibodies treatment did not reduce the amount of galectin-8 adsorbed on the plates (not shown). All plates were washed, and further blocked (2 hours at 37°C) with RPMI containing 10% fetal calf serum. 3x10^5 1299 cells were then added, with or without 10 mM TDG, as indicated. After a 2 hour incubation at 37°C, the wells were washed and the number of adherent cells was determined.
forms of complex cell surface carbohydrates, that fail to interact effectively with other galectins.

**Tissue distribution and secretion of galectin-8**

Galectin-8 is expressed in rat liver, muscle and kidney. Low or barely detectable levels of expression were detected in rat intestine, and L-8 and MDCK cells (Fig. 3A) as well as testis, fat, thymus and lung (not shown). These results demonstrate that although galectin-8 is widely expressed, it is confined to certain specific tissues. To determine whether galectin-8 could act extracellularly, we studied whether, like other galectins (Hughes, 1999), galectin-8 is a secreted protein. Two forms of immunoreactive galectin-8 were detected in whole homogenates of 1299 cells (Fig. 3B). When 24 and 48 hour-conditioned medium of 1299 cells was collected, precipitated, resolved by SDS-PAGE and immunoblotted with galectin-8 specific antibodies, we could demonstrate that the slow-migrating form of galectin-8-immunoreactivity accumulated in the extracellular medium in a time-dependent manner (Fig. 3). A larger amount of the protein was present in the 48 hour-conditioned medium, compared to the amount detected by 24 hours, suggesting that galectin-8, like other galectins, is secreted and could act extracellularly.

Additional support for this was obtained from trypsinization experiments. As shown in Fig. 4, trypsinization of intact 1299 cells resulted in >55% reduction in the amount of cell-associated galectin-8, with no reduction in the amount of Grb-2, a known cytosolic protein. Both galectin-8 and Grb-2 were readily degraded once trypsin was added to cell homogenates (not shown). These findings indicate that extracellular trypsinization did not degrade intracellular proteins and suggest that a significant fraction of the secreted galectin-8 remains bound to the extracellular surface.

**Inhibition of cell adhesion, induced by galectin-8, is not due to steric hindrance of cell-matrix interactions**

Galectin-8 could inhibit cell adhesion by binding to the extracellular matrix (ECM), thus creating a steric hindrance of cell-matrix interactions. Such a mechanism has been suggested to account for the inhibitory action of other mammalian or plant lectins (Bouzon et al., 1990; Cooper et al., 1991; Dean et al., 1990; Sato and Hughes, 1992). Alternatively, galectin-8

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**Fig. 2.** The inhibitory effects of galectin-8 on cell adhesion are specific. (A) A suspension (3x10^5/ml) of 1299 was made in RPMI medium containing 10% fetal calf serum. Cells were seeded in 24-well Costar plates in the absence or in the presence of 1 μM of the indicated lectin. Following a 2 hour incubation at 37°C, non-adherent cells were washed, and adherent cells were collected and counted. (B) Plates were coated with 1 ml solution containing 1 μM of the indicated lectin as described in the legend to Fig. 1D. Following the first coating plates were washed, and further blocked (2 hours at 37°C) with RPMI containing 10% fetal calf serum. 3x10^5 1299 cells were then added. After a 2 hour incubation at 37°C, the wells were washed and the number of adherent cells was determined. Values are means ± s.d. (n=4).

**Fig. 3.** Tissue distribution and secretion of galectin-8. (A) Different rat tissues, as well as Fao, MDCK and L-8 cell lines, were extracted in buffer A. 100 μg protein from each tissue were resolved on 12% SDS-PAGE. The proteins were transferred to nitrocellulose papers and western immunoblotted using anti-galectin-8-specific antibodies. (B) 1299 cells, grown to confluence on 10 cm plates, were incubated for 24 or 48 hours in serum-free medium. At the end of the incubation the cells were extracted in buffer A. The media were collected and the proteins were precipitated with DOC/TCA as described in Materials and Methods. 100 μg protein from the total cell extracts (two left lanes), and 100 μl of the proteins from the resuspended media (two right lanes) were resolved by 12% SDS-PAGE, transferred to nitrocellulose paper and western immunoblotted with galectin-8 specific antibodies.
could play an active role in negatively regulating cell-matrix interactions, for example, by binding to integrin receptors (Hynes, 1992) and inducing a conformational change. To distinguish between these possibilities, antibodies directed against galectin-8 were added to plates already coated with galectin-8, and their effect on cell adhesion was evaluated. If galectin-8 were creating a steric hindrance of cell-matrix interactions, the inclusion of antibodies should have no effect or even further inhibit adhesion. However, inhibition was reversed when galectin-8-coated plates were further coated with galectin-8 antibodies, or when the cells were plated in the presence of 10 mM TDG (Fig. 1D). In control experiments we could demonstrate that TDG did not simply release galectin-8 from the plates since precoating of plates with galectin-8 in the presence of TDG did not abolish the inhibitory effects of the lectin, as long as TDG was washed away prior to addition of the cells (Fig. 1D). Taken together, these results suggested that the inhibitory effects of galectin-8 were not due to non-specific sterical hindrance which might prevent interactions of the cells with the matrix ligands.

Galectin-8 selectively inhibits cell adhesion to integrin ligands

To better characterize the nature of inhibition exerted by galectin-8 it was of interest to examine its effects on cell adhesion to various matrices. Addition of galectin-8 to a suspension of 1299 cells markedly inhibited their adhesion to plates coated with laminin, fibronectin (FN), or the 120 kDa proteolytic fragment of FN (FN-120), which includes its integrin-binding domains (Potts and Campbell, 1994) (Fig. 1C). In contrast, galectin-8 did not affect cell adhesion to polylysine-coated plates, suggesting that it specifically inhibits integrin-mediated cell adhesion but not a non-specific adhesion, mediated by electrostatic interactions with polylysine.

To further evaluate the role of integrins in mediating the inhibitory effects of galectin-8, the effects of Mn$^{2+}$ were studied. Mn$^{2+}$ increases the affinity of integrins for their ligands, independent of inside-out signals, thus maintaining them in their high-affinity state (Hynes, 1992; Stuiver and O’Toole, 1995). As shown in Fig. 1C, inclusion of Mn$^{2+}$ eliminated the inhibitory effects exerted by galectin-8, thus lending further support to the notion that galectin-8 might exert its down-modulatory effects on adhesion by interacting with cell surface integrins.

Galectin-8 induces an apoptotic process

Impairment of matrix-integrins interactions has been implicated in the induction of apoptosis (Cadone et al., 1997; Frisch and Francis, 1994; Ruoslahti and Reed, 1994). We have therefore investigated whether galectin-8 could induce an apoptotic process. As shown in Fig. 5A,B, addition of galectin-8 to adherent 1299 cells maintained in serum-free medium, induced apoptosis in more than 50% of the cells following a 6 hour incubation with the lectin. This was evident by their DNA content of $<$2N that fell into the sub-G1 fraction. Reduction in DNA content was accompanied by the appearance of condensed and fragmented nuclei (Fig. 5C), a hallmark of an apoptotic process. Apoptosis was not secondary to the anti-adhesive effects of galectin-8, since galectin-8 did not induce detachment of the (already adherent) cells within the time frame of the experiment (6 hours). Apoptosis was prevented upon inclusion of TDG, suggesting that both inhibition of cell adhesion and apoptosis were mediated by interactions of galectin-8 with cell surface glycoconjugates. Nonetheless, in contrast to inhibition of cell adhesion, which occurred in the presence of serum, apoptosis induced by galectin-8 occurred only in a serum-free medium, suggesting that serum constituents presumably act as survival factors to rescue the cells from apoptotic signals transmitted by galectin-8 (Fig. 5A,B). Notably, apoptosis induced by galectin-8 is p53-independent, since 1299 cells have lost any functional p53 (Mitsudomi et al., 1992).

Galectin-8 inhibits colony formation upon transfection into 1299 cells

To assess its physiological role in regulating cell-matrix interactions and apoptosis, 1299 cells were transfected with a cDNA encoding rat galectin-8 in the sense or anti-sense orientation. Galectin-8 transfection significantly reduced (approx. 75%) the number of G-418 resistant colonies, compared with empty vector-transfected cells (Fig. 6). This effect was specific since no reduction in the number of colonies was detected when cells were transfected with a vector containing galectin-8 cDNA in the anti-sense orientation (Fig. 6). These findings therefore suggested that a secreted galectin-8 might act extracellularly to inhibit colony formation.

$\alpha_\beta_1$ integrin is a major cellular constituent that binds immobilized galectin-8

Galectin-8 may induce apoptosis and inhibit colonies formation through interaction with cell surface integrins that regulate both processes (Akiyama et al., 1994; Ruoslahti and Reed, 1994). To further explore this possibility we studied the nature of galectin-8 binding-proteins derived from 35S-labeled extracts of 1299 cells. A single major protein, having a molecular mass of 125 kDa, was retained by recombinant glutathione-S-transferase (GST)-galectin-8, immobilized on.
glutathione-agarose beads (Fig. 7, left). Amino-terminal sequencing of the bound protein revealed the sequence FNLDTRFL VVK, which is identical to the N-terminal sequence of integrin \(\alpha_3\) (\(\alpha_3\)), save for an F to D substitution at position 4. Western blot analysis with anti-\(\alpha_3\) antibodies (Fig. 8A) confirmed the identity of the bound protein as the \(\alpha_3\) subunit of the integrin receptors, which is expressed on the surface of 1299 cells (see below). Although \(\alpha_3\) integrin was a major protein that interacted with immobilized galectin-8, western blot with antibodies directed against the \(\beta_1\) subunit of integrins revealed that this subunit was also retained by the immobilized lectin (Fig. 8A). These findings support the notion that galectin-8 interacts with the heterodimeric (\(\alpha_3\beta_1\)) form of integrin receptors. Binding involved protein-sugar interactions, since it was eliminated in the presence of 10 mM TDG (Fig. 7, left). Binding of galectin-8 was not restricted to \(\alpha_3\beta_1\) integrin derived from 1299 cells. As shown in Fig. 8A, immobilized galectin-8 effectively bound the \(\alpha_3\) and \(\beta_1\) subunits of integrins derived from HeLa cells, Granulosa cells and human endothelial cells.

**Galectin-8 interacts with \(\alpha_3\beta_1\) integrin within the physiological context of intact cells**

The above findings already suggested that \(\alpha_3\beta_1\) integrin could form stable complexes with galectin-8. To assess the physiological significance of these interactions, binding of galectin-8 to \(\alpha_3\beta_1\) was studied within the context of intact cells. As shown in Fig. 9, addition of rGalectin-8 to intact 1299 cells inhibited subsequent binding of antibodies directed against the extracellular regions of \(\alpha_3\) or \(\beta_1\) integrins. FACS analysis indicated that pre-incubation with galectin-8, blocked subsequent binding of antibodies directed towards integrin \(\alpha_3\) and integrin \(\beta_1\) by approx. 50% and 60%, respectively (not shown). In contrast, binding of antibodies directed towards the extracellular region of Erb-2, a member of the EGF receptor family, was not affected by the presence of galectin-8.

More importantly, galectin-8 coprecipitated with \(\alpha_3\beta_1\) integrin when extracts derived from native 1299 cells were subjected to immunoprecipitation with polyclonal \(\alpha_3\)-antibodies (directed to the intracellular domain of \(\alpha_3\)) (Fig. 7, right). Coimmunoprecipitation of galectin-8 and \(\alpha_3\beta_1\) was specific and did not occur when normal serum or irrelevant antibodies were used (Fig. 7, right).

**Integrin binding-specificity of galectin-8**

To further explore the capability of galectin-8 to interact with other members of the integrin family, cells extracts were incubated with immobilized galectin-8, and integrins that remained bound were detected by western blotting. As shown in Fig. 8B, galectin-8 effectively bound the \(\alpha_6\) subunit of integrins derived from extracts of HeLa cells, while its ability to specifically bind the \(\alpha_4\) subunit of integrins, derived from HeLa cells, 1299, or human endothelial cells, was significantly lower. Similarly, only trivial amounts of the \(\beta_3\) subunit of integrins were retained by galectin-8 (Fig. 8C). Quantitative densitometry revealed that galectin-8 bound 60%, 56% and 23% of the \(\alpha_6\), \(\alpha_3\) and \(\beta_1\) integrin subunits present (respectively) in 1 mg of extracts of HeLa cells (Fig. 8). In contrast, only 1.5% of the \(\alpha_4\) integrin subunits present in the same extracts were retained by the immobilized lectin. Similarly, 26% and 18% of the \(\alpha_3\) and \(\beta_1\) integrins (respectively) present in 1 mg of extracts of human endothelial

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**Fig. 5.** Apoptosis induced by galectin-8. Adherent 1299 cells were incubated for 6 hours in serum-free RPMI medium in the absence or presence of 0.5 \(\mu\)M galectin-8, or 0.5 \(\mu\)M galectin-8 and 10 mM TDG. Some cells, as indicated, were incubated with 0.5 \(\mu\)M galectin-8 in RPMI containing 10% FCS. Following treatment, cells were fixed with methanol, and analyzed by flow cytometry. (A) The distribution of DNA content as a result of the different treatments. (B) Percentage of the sub-G1 subpopulation following each treatment. (C) DAPI staining of control and galectin-8-treated cells.
cells remained bound to galectin-8 (Fig. 8), while only minute amounts (0.4%) of the β3 subunit bound galectin-8. Consistent with these findings galectin-8 had only limited effects on adhesion of BAE (bovine aorta endothelial) cells to vitronectin. Adhesion of these cells to vitronectin was inhibited approx. 25% in the presence of galectin-8 and this small inhibition was insensitive to the presence of the sugar TDG (not shown). These results are not surprising in view of the fact that adhesion of BAE cells is mainly mediated by integrin αvβ3, which does not interact with galectin-8. Collectively, these findings implicate the extracellular domain of specific integrins like αv, α3 and β1 as key cellular components that selectively bind galectin-8, and places these integrins as potential receptors that could mediate the anti-adhesive effects of galectin-8.

DISCUSSION

In the present study we provide structural and functional evidence suggesting that galectin-8, a widely expressed mammalian lectin, selectively interacts with a particular subgroup of cell surface integrins. Interaction of a secreted galectin with integrins offers a novel mechanism of generating anti-adhesive signals that modulate cell-matrix interactions.

Lectins of different types, including galectins, have already been shown to inhibit cell-matrix interactions, but these effects were attributed merely to direct interactions of the lectins with sugar moieties of matrix proteins (Bouzon et al., 1990; Cooper et al., 1991; Dean et al., 1990; Sato and Hughes, 1992). Galectins, by virtue of their affinity for glycans carrying multiple N-acetyl lactosamine units, bind to certain isoforms of laminin and fibronectin that express such glycans (Sato and Hughes, 1992). These lectin-ECM interactions presumably generate a steric hindrance that masks cell-binding sites for integrins and other adhesion receptors on the ECM. In contrast, our results suggest that galectin-8 does not interact primarily with cell recognition sites on matrix proteins to generate a steric hindrance. Instead, we propose that galectin-8 acts as an integrin binding-protein that exerts down-modulatory effects on integrin receptor functions.

Several lines of evidence support this idea. First, galectin-8 inhibits cell adhesion when immobilized on culture plates, and this inhibition is reversed when galectin-8-coated plates are further coated with galectin-8 antibodies. If the immobilized galectin-8 were just creating a steric hindrance that masks cell-binding sites for integrins and other adhesion receptors on the ECM. In contrast, our results suggest that galectin-8 does not interact primarily with cell recognition sites on matrix proteins to generate a steric hindrance. Instead, we propose that galectin-8 acts as an integrin binding-protein that exerts down-modulatory effects on integrin receptor functions.

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and should not relieve the inhibition. Instead, the ability of the sugar to relieve the inhibition strongly suggests that galectin-8 does, in fact, induce anti-adhesive effects. It should be noted that galectin-8 is immobilized on the culture plates through non-specific interactions with the plastic. These interactions do not involve the sugar-binding sites of the protein since galectin-8 is not detached from the plates upon addition of TDG. Similarly, addition of galectin-8 antibodies does not strip the immobilized lectin off the plates. Hence, when plates are coated with galectin-8, the seeded cells are still able to maintain normal adhesion, as long as they are not generating protein-sugar interactions with galectin-8. This leads us to propose that galectin-8 inhibits cell adhesion through interactions with cellular components, whose binding to this lectin impedes the normal process of cell adhesion.

The anti-adhesive effects induced by galectin-8 are specific. Failure to inhibit cell adhesion by other galectins, or plant lectins known to interact with galactose/N-acetylgalactosamine, suggests that either the sugar binding specificity of galectin-8, which somewhat differs from other galectins, is sufficient to induce its specific biological functions or, more likely, that the interaction of galectin-8 with cellular proteins is mediated by a combination of both protein-protein and protein-sugar interactions. Computer modeling of the three-dimensional structure of galectin-8 supports the theory that the N-terminal carbohydrate recognition domain (N-CRD) of galectin-8 deviates from the canonical structure of sugar binding sites of galectins. This could be attributed to the presence of Ile90 within a WG-E-I motif, in the N-CRD of galectin-8 (Hadari et al., 1997a, 1995), which replaces a conserved basic residue that occupies this site in all other galectins, including the C-terminal CRD of galectin-8 itself. The presence of a modified sugar-binding site at the N-CRD, and the unique sequences that flank this region (Hadari et al., 1997a, 1995), could confer upon galectin-8 its ability to interact with specific regions within the extracellular domains of integrins and inhibit cell adhesion.

Our data suggests that integrins are major cellular components that interact with galectin-8. When added to a suspension of 1299 cells, galectin-8 selectively inhibits cell adhesion to plates coated with integrin ligands laminin and fibronectin, while it fails to inhibit cell adhesion to the non-specific substratum polylysine. Furthermore, the inhibitory effects of galectin-8 are reversed upon inclusion of Mn$^{2+}$, which compensates for any deficit in integrin function (Hynes, 1992; Stuiver and O’Toole, 1995). Our results are in agreement with previous findings (Gu et al., 1994) indicating that galectin-1 interacts, at least in vitro, with integrins ($\alpha\beta_{1}$) expressed on the surface of differentiating myoblasts. A key difference between the previous observations and those described here is that galectin-1 effectively inhibits adhesion of myoblasts to laminin, while it fails to inhibit the interactions of $\alpha\beta_{1}$ integrins with fibronectin. In contrast, our results clearly indicate that galectin-8 inhibits cell adhesion to different integrin ligands, including fibronectin. Hence, different galectins might selectively regulate interactions of integrins with matrix proteins. While galectin-8 seems to be a potent modulator of cell adhesion to different matrices, galectin-1 might interact with integrins in a somewhat different fashion, which results in selective interference with laminin-integrin interactions.

Although $\alpha_3\beta_1$ integrin, retained by immobilized galectin-8, is a major cellular constituent expressed on the surface of 1299 cells, galectin-8 also interacts with other integrins to induce its anti-adhesive functions. Indeed, the $\beta_1$ subunit of integrins, extracted from different cell types, was retained by immobilized galectin-8, and galectin-8 inhibited binding of $\beta_1$ antibodies to intact cells. Similarly, galectin-8 effectively interacts with the $\alpha_6$ subunit of integrins. In contrast, galectin-8 maintains only minimal, if not trivial, interactions with integrins $\alpha_4$ and $\beta_3$. These findings implicate the extracellular domain of specific integrins like $\alpha_6$, $\alpha_3$ and $\beta_1$ as key cellular components that selectively bind galectin-8, and places these integrins as potential receptors that could mediate the anti-adhesive effects of galectin-8. This conclusion is supported by the fact that galectin-8 inhibits adhesion of other cell types (e.g. Hep-G2 and HAKAT cells) that express integrin $\alpha_6$ at equal or even higher amounts than integrin $\alpha_3$. Conversely, galectin-8 has only limited effects on adhesion of BAE cells to vitronectin. These results are expected in view of the fact that

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**Fig. 8.** Binding of integrins to GST-galectin-8. Extracts of the indicated cell types were centrifuged for 15 minutes at 14,000 g at 4°C and the supernatants (1 mg) were incubated at 4°C for 2 hours with immobilized GST or GST-galectin-8. Following incubation, the beads were washed and bound proteins were extracted and subjected to SDS-PAGE and western immunoblotting with (A) anti-$\alpha_3$ or $\beta_1$ integrin antibodies, (B) anti-$\alpha_4$ or $\alpha_6$ integrin antibodies, (C) anti-$\alpha_3$, -$\beta_1$ or $\beta_3$ integrin antibodies, as indicated.
adhesion of BAE cells to vitronectin is mainly mediated by integrin \( \alpha_3\beta_1 \), which does not interact with galectin-8. Collectively, these findings implicate the extracellular domain of specific integrins like \( \alpha_6 \), \( \alpha_3 \) and \( \beta_1 \) as galectin-8-binding sites, and suggests that these integrins could mediate the anti-adhesive effects of galectin-8. The fact that Mn\(^{2+} \), a promoter of integrin function (Hynes, 1992), reverses the inhibitory effects of the lectin, supports our theory that integrins are indeed the key mediators of galectin-8 inhibitory functions on cell adhesion.

Interaction of galectin-8 with integrins seems to be of physiological relevance. Immunofluorescence and immunohistochemical studies revealed that addition of rGalectin-8 to intact 1299 cells inhibited subsequent binding of \( \alpha_3 \)- or \( \beta_1 \)-antibodies, directed towards the extracellular region of these proteins (Fig. 9), while inclusion of galectin-8 antibodies eliminated this inhibitory effect (Goren et al., unpublished observations). These observations indicate that binding of galectin-8 to the cells masks essential antigenic determinants on the integrin surface, and supports the notion that galectin-8 interacts with integrins present in their native milieu within the context of an intact cell. Indeed, galectin-8 remained bound to \( \alpha_3\beta_1 \) when 1299 cell extracts were subjected to immunoprecipitation with \( \alpha_3 \)-antibodies directed to the intracellular region of \( \alpha_3 \) (Fig. 7, right). In contrast, binding of Erb-2 antibodies to the extracellular domain of the Erb-2 receptor was not affected by the presence of the galectin-8, thus emphasizing the selectivity of this effect. Our results suggest that a secreted form of galectin-8 might act in an autocrine fashion when it remains bound to the extracellular regions of integrins expressed on the surface of 1299 cells. This conclusion is supported by the fact that trypsinization of intact 1299 cells reduces by >55% the amount of cell-associated galectin-8, while it does not affect the cellular content of intracellular proteins. The inhibitory effects of immobilized galectin-8 (Fig. 1D) lend further support to the idea that galectin-8 presumably acts as an external ligand that exerts its inhibitory effects upon binding to cell surface receptors.

Collectively, our findings implicate integrins as major cellular components through which a secreted galectin-8 manifests its biological activities. In line with this idea are the experiments demonstrating inhibition of colony formation in cells transfected with the gene coding for galectin-8. The inhibitory effects of the overexpressed lectin could be accounted for by an autocrine effect of the secreted lectin that interacts with the available cell surface integrins, thus preventing cell adhesion altogether, similar to its anti-inhibitory role when added exogenously. Several mechanisms could account for the inhibition of cell adhesion as a result of integrin-galectin-8 interactions. Binding of galectin-8 to integrins could generate a complex that prevents integrins from further interactions with other ECM proteins. If this were the case than inclusion of Mn\(^{2+} \), which compensates for any deficit in integrin function (Hynes, 1992; Stuiver and O’Toole, 1995) would not be expected to relieve the steric inhibition of the lectin. Although we cannot rule out the possibility that Mn\(^{2+} \) maintains integrins in a conformation that is inaccessible to the lectin, preliminary studies reveal that Mn\(^{2+} \) does not affect the ability of \( \alpha_3\beta_1 \) integrin to interact with immobilized galectin-8. Hence, a trivial steric hindrance induced upon formation of galectin-integrin complexes is unlikely to occur. Alternatively, galectin-8 binding to integrins could induce the latter to maintain their low-affinity conformation, independent of other signaling cues. This type of inhibition could be regarded as an opposite mechanism of integrin activation by extracellular ions or certain monoclonal anti-integrin antibodies, which is caused by a direct conformational change of the integrin molecule (Ginsberg et al., 1992). Rather than directly inducing a low-affinity state, inactivation of integrins could be mediated by an ‘inside-out signal’, initiated upon binding of galectin-8 to integrin receptors. Several signal transduction pathways were
implicated in mediating anti-adhesive processes triggered upon ligation of integrin receptors. These could involve phosphorylation of the β1-integrin cytoplasmic domain by a β1-integrin-linked Ser/Thr kinase (p59flik) (Hannigan et al., 1996), or could be mediated, for example, by a Ras/Raf-initiated MAP kinase pathway that suppresses integrin activation (Hughes et al., 1997).

The role of α3 integrin in mediating cell adhesion has been studied extensively. It could be demonstrated that α3β1 functions as a major integrin receptor for collagen, laminin and fibronectin in small cell lung carcinoma line (NCI-H69), which, similar to the 1299 cells, expresses high levels of α3 (Elices et al., 1991). Interaction of α3β1 with these ligands occurred in both an RGD-dependent and RGD-independent manner. α3β1 integrin has also been implicated in heterophilic interactions with α3β1 (Symington et al., 1993). Moreover, α3β1 has been proposed to function as a secondary receptor with post-cell adhesion functions for a broad spectrum of extracellular matrices (DiPersio et al., 1995). Collectively, these observations suggest that α3β1 could function as a major integrin receptor in 1299 cells, whose occupancy by galectin-8 inhibits cell adhesion. Similarly, other integrins such as α3β1 could be the targets for interactions with galectin-8 in other cell types such as HeLa and Hakat cells. Signalling events initiated by the occupancy of integrins by galectin-8 could suppress functions associated with other integrins that act with them in concert, a process known as integrin crosstalk (Blystone et al., 1994) or trans-dominant inhibition (Diaz-Gonzalez et al., 1996; Huhtala et al., 1995).

Inhibition of matrix-integrins interactions has been implicated in the induction of apoptosis in cells denied of anchorage (Frisch and Francis, 1994; Ruoslahti and Reed, 1994). We could demonstrate that galectin-8 is an effective inducer of an apoptotic process in 1299 cells. Apoptosis, like binding to integrins, was prevented upon inclusion of TDG, suggesting that both events were at least partially mediated by interactions of galectin-8 with cell surface glycoconjugates. However, apoptosis induced by galectin-8 was not secondary to its anti-adhesive signals, since it took place when galectin-8 was applied to adherent cells that did not detach during the course of the experiment. Moreover, and in contrast to inhibition of cell adhesion, which occurred in the presence of serum, apoptosis induced by galectin-8 occurred only in a serum-free medium. These findings suggest that galectin-8 might induce its anti-adhesive and apoptotic effects by two independent mechanisms; furthermore, serum constituents might act as survival factors to rescue the cells from apoptotic signals transmitted by galectin-8. Of note is the fact that apoptosis induced by galectin-8 differs from that induced by galectin-1 (Perillo et al., 1995), which is serum-independent, again suggesting that these two lectins might act through two different mechanisms.

Notably, galectins are present in tumor cells intra- and extracellularly. Galectin-3 is overexpressed in human colon and gastric carcinomas (Raz and Lotan, 1987), whereas prostate carcinoma tumor antigen-1, the human isoform of galectin-8 (Su et al., 1996), is selectively expressed in prostate carcinoma (Su et al., 1996). Because interactions of a secreted galectin-8 with cell-surface integrins inhibits cell adhesion, galectin-8 may modulate cell-matrix interactions in a variety of pathological processes.

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