Galectin-3 Regulates Integrin $\alpha_2\beta_1$-mediated Adhesion to Collagen-I and -IV*

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Galectins are a taxonomically widespread family of galactose-binding proteins of which galectin-3 is known to modulate cell adhesion. Using single cell force spectroscopy, the contribution of galectin-3 to the adhesion of Madin-Darby canine kidney (MDCK) cells to different extracellular matrix proteins was investigated. When adhering to collagen-I or -IV, some cells rapidly entered an enhanced adhesion state, marked by a significant increase in the force required for cell detachment. Galectin-3-depleted cells had an increased probability of entering the enhanced adhesion state. Adhesion enhancement was specific to integrin $\alpha_2\beta_1$, as it was not observed when cells adhered to extracellular matrix substrates by other integrins. The adhesion phenotype of galectin-3-depleted cells was mimicked in a galactoside-deficient MDCK cell line and could be complemented by the addition of recombinant galectin-3. We propose that galectin-3 influences integrin $\alpha_2\beta_1$-mediated adhesion complex formation by altering receptor clustering.

The adhesion of cells to the extracellular matrix (ECM)\(^2\) is essential for various physiological and pathological processes. Cell-ECM interactions are mediated by numerous adhesion receptors, of which integrins are the most prominent. Following initial adhesion to the ECM, integrins cluster and proteins are recruited to the newly formed contact sites, forming focal complexes, focal adhesions, and fibrillar adhesions. The formation of cytoplasmic protein assemblies anchors the adhesion complexes to the actin cytoskeleton and activates intracellular signaling cascades that control cellular responses (1–3). Although this regulation is complex, two main types: protein-protein and protein-carbohydrate interactions mediate specific adhesion. Galectins have been implicated in the latter category.

Galectins are a family of lectins, characterized by a common carbohydrate recognition domain (CRD) and affinity for $\beta$-galactosides. They are widely distributed in vertebrates and invertebrates; including nematodes, insects, sponges, and fungi (4). In mammals, galectins occur at high concentrations only in a few cell types. Galectins lack signal sequences and, thus, are synthesized in the cytoplasm. They are not only found in the cytoplasm, in the nucleus and along the cell cortex, but also extracellularly, as they can be secreted via a non-classical pathway (5). Given their diverse distribution, it is not surprising that galectins have been implicated in a variety of biological processes; including development, differentiation, morphogenesis, tumor metastasis, apoptosis, and RNA splicing (6). Unfortunately, relatively little is known about the mechanisms by which galectins influence these processes.

The fourteen human galectins are classified into three categories: prototype, tandem repeat, and chimeric. Prototype galectins contain one CRD and exist either as monomers or non-covalent homodimers. Tandem repeat galectins consist of two distinct CRDs joined by a linker peptide. Galectin-3 is the only chimeric galectin (7).

Galectin-3 is expressed in macrophages, activated T-cells, epithelial cells, fibroblasts, and often in tumor cells. On the cell surface, galectin-3 associates with a large number of different glycoconjugates including integrins and ECM proteins (8). Galectin-3 consists of a C-terminal CRD and an N-terminal collagen-like domain. Through intermolecular interactions via the collagen-like domain, galectin-3 self-associates into multimers (9). Thus, galectin-3 is able to cross-link cell surface glycoproteins, thereby activating cells or mediating cell-cell and cell-ECM adhesion (10, 11). Depending on the cell type studied and the experimental conditions used, galectin-3 has been shown to both promote and inhibit cell adhesion (12). Because many of these studies reported the effects of high concentrations of exogenous, recombinant galectin-3, the physiological functions of this lectin remain unclear.

In this study, we applied single cell force spectroscopy (SCFS) to determine the role of galectin-3 in early adhesion of MDCK cells to various ECM constituents. Reducing galectin-3 expression did not alter early adhesion to fibronectin or laminin-332. However, on collagen-I and -IV galectin-3 knockdown cells had an elevated probability of entering an enhanced adhesion state. This was dependent on integrin $\alpha_2\beta_1$ and characterized by a significant increase in the force required for cell detachment. We deduced that galectin-3 influences $\alpha_2\beta_1$-mediated adhesion to regulate cooperative receptor binding.
**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MDCK strain II cells were maintained in MEM supplemented with 5% fetal calf serum, 2 mM L-glutamine, and 100 units/ml penicillin/100 μg/ml streptomycin (growth medium; all Invitrogen). The retrovirus-mediated knockdown of galectin-3 and -9 has been described previously (13). Briefly, target sequences corresponding to the canine galectin-3 (528–548) and galectin-9 (867–887) coding sequences were selected. Annealed oligonucleotides were cloned into an RVH-1-puro retroviral vector and recombinant knockdown viruses were generated. MDCK strain II cultures were infected and incubated for 8–12 h. Infection was repeated 1–2 times before trypsinizing and re-seeding cells in MEM containing puromycin (4 μg/ml; BD Biosciences). Knockdown cells were used for up to 2 weeks without a significant drop in knockdown efficiency (14). MDCK cells infected with retroviruses lacking a target sequence were used as controls. Twelve hours prior to SCFS experiments, the medium was replaced with CO₂-independent medium (Invitrogen) supplemented with L-glutamine, penicillin/streptomycin, and 1% fetal calf serum. For SCFS experiments, cells were incubated with PBS, trypsinized, pelleted, and resuspended into serum-free CO₂-independent medium (measurement medium). Cells recovered for 15 min at 37 °C under constant rotation. Antibodies AllIB2 (1:10 dilution of hybridoma supernatant; kind gift of K. Matlin, Department of Surgery, University of Chicago, Chicago, IL), GoH3 (5 μg/ml; BD Pharmingen) and IgG from rat serum (10 μg/ml; Invitrogen) as well as RGD peptides (100 μg/ml; Sigma), RGE peptides (100 μg/ml; Coring Diagnostix GmbH), full-length galectin-3 (4 μg/ml), and galectin-3-CRD (4 μg/ml; corresponding to residues 114–250 of human galectin-3) were incubated together with cells on ice for 30 min prior to SCFS experiments.

**Epifluorescent Detection of Endogenous Galectin-3**—For detection of extra- and intracellular galectin-3, MDCK cells were seeded on poly-L-lysine (Sigma)-coated coverslips and adhered for 20 min. Cells were fixed for 20 min in ice-cold paraformaldehyde (4%/PBS). After a PBS wash, residual aldehyde was quenched for 20 min in PBS containing 200 mM glycine, pH 9.2) was added to freshly cleaved mica and residual air plasma cleaned for 1 min. Then, cantilevers were incubated at 4 °C in 2 mg/ml concanavalin A (ConA; from *Canavalia ensiformis*, Sigma) overnight. Glass coverslips (24 mm) were coated as follows. After sequential washes in 1 N HCl, water, and EtOH, coverslips were air-dried and then, incubated in 0.8 μg/ml rat laminin-332 (Chemicon) for 2 h at 37 °C, 50 μg/ml human plasma fibronectin (Roche) for 2 h at 25 °C or 20 μg/ml collagen-IV (Sigma) for 12 h at 4 °C. For collagen-I coating, mica discs (6.5 mm) were glued (OP-29, Dymax Europe GmbH) to glass coverslips (24 mm). Then, bovine dermal collagen (30 μg/ml, Cohesion) in coating buffer (200 mM KCl, 50 mM glycine, pH 9.2) was added to freshly cleaved mica and incubated overnight at room temperature (17). Unbound protein was removed with PBS and fresh measurement medium.

**Cell Capture, Force Measurement, and Data Processing**—For SCFS experiments, cells suspensions were pipetted into the BioCell containing a coated coverslip. The apex of a ConA-coated cantilever was lowered onto a cell until a force of 0.75 nN was applied to the cell. After a contact time of 5 s, the cantilever was moved 50 μm from the surface where the cantilever-bound cell recovered for >5 min. For force-distance curve measurements, the approach and retract rates were 5 μm/s and the contact force 0.75 nN. The pulling range was 50 μm. At a contact time of 20 s, 10–15 force-distance curves were recorded with >20 s resting periods between approach/retract cycles. Cells were allowed to recover for ~5 min, before a new set of force-distance curves was recorded. At 90 s contact time, force-distance curves were recorded at 3-min intervals, never exceeding 5 force-distance curves per cell. Loss of adhesion strength with force-distance curve cycles could not be observed (data not shown). Detachment forces and force step sizes were extracted using in-house algorithms in Igor Pro 5.05A (Wavemetrics). InStat (GraphPad Software) was used for running the Student’s *t* test. All error bars show S.D. unless stated otherwise.

**TIRF Experiments**—To analyze the contact area of MDCK cells with collagen-IV supports, a combined AFM-total internal reflection fluorescence (TIRF) microscopy setup was used. The AFM-bearing light microscope was additionally outfitted with a Laser TIRF Slider (Zeiss) coupled over a multi-mode optical fiber (AMS Technologies) to a Sapphire 488–50 cdhr laser (Coherent). Images were acquired with a Coolsnap cf camera (Roper Scientific) and a ×100/1.45 alpha Plan-FLUAR oil objective (Zeiss). A FITC filter set (Chroma Technology Corp.) was used. Cells were trypsinized, washed once with growth medium without fetal calf serum, pelleted, and incubated with 20 μg/ml of a cell-labeling reagent (PKH67, Sigma) for 4 min at 25 °C. Then, cells were extensively washed with growth medium before they were resuspended in measurement medium.

**Galectin-3 Influences Integrin α₂β₁ Cooperativity**

with the CellHesion module (JPK Instruments) (15). A BioCell (JPK Instruments) allowed measurements to be conducted at 37 °C. Cantilevers used were 200-μm long V-shaped silicon nitride tipless cantilevers with nominal spring constants of 0.06 N/m (NP-0, Veeco). Spring constants were calibrated using routines based on the equipartition theorem (16).

**Surface Coating of AFM Cantilever and Coverslips**—AFM cantilevers were soaked in 10% Hellmanex (Hellma GmbH) and residual air plasma cleaned for 1 min. Then, cantilevers were incubated at 4 °C in 2 mg/ml concanavalin A (ConA; from *Canavalia ensiformis*, Sigma) overnight. Glass coverslips (24 mm) were coated as follows. After sequential washes in 1 N HCl, water, and EtOH, coverslips were air-dried and then, incubated in 0.8 μg/ml rat laminin-332 (Chemicon) for 2 h at 37 °C, 50 μg/ml human plasma fibronectin (Roche) for 2 h at 25 °C or 20 μg/ml collagen-IV (Sigma) for 12 h at 4 °C. For collagen-I coating, mica discs (6.5 mm) were glued (OP-29, Dymax Europe GmbH) to glass coverslips (24 mm). Then, bovine dermal collagen (30 μg/ml, Cohesion) in coating buffer (200 mM KCl, 50 mM glycine, pH 9.2) was added to freshly cleaved mica and incubated overnight at room temperature (17). Unbound protein was removed with PBS and fresh measurement medium.
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Protein Expression and Purification—Full-length, human galectin-3 cDNA was expressed in Escherichia coli BL21 cells, using the pET15b expression vector (kind gift of K. Simons, MPI-CBG, Dresden). Galectin-3 was purified from bacterial cell lysates using unmodified Sepharose (Sigma). In detail, after transforming electro-competent BL21 cells with the expression vector, a positive clone was grown at 37 °C until A₆₀₀ had reached 0.8. The culture was diluted with ice-cold LB medium and expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (Carl Roth) to a final concentration of 0.4 mM. Then, the cultures were incubated at 18 °C for 12–16 h. Cells were harvested by centrifugation (20 min, 2000 × g), resuspended in 1:20 volume of Buffer A (20 mM Tris- HCl, 5 mM EDTA, 200 mM NaCl, 1 mM dithiothreitol, protease inhibitors (Roche, Complete Mini)), and lysed by sonication (100 watts) at 4 °C over six 10-s pulses separated by 30-s intervals. The lysate was clarified at 15,000 × g for 15 min at 4 °C. Protein was purified from the lysate by affinity chromatography in a column containing 7 ml of Sepharose (Sigma) and washed with several column volumes of Buffer A. Elution was performed using Buffer B (Buffer A + 200 mM lactose), collecting fractions of 1 ml volume. Based on SDS-PAGE profiles, the peak fractions (usually 2–4 fractions) were pooled. The pools were concentrated by passage through a Centricon™ microconcentrator (Millipore), followed by extensive dialysis against PBS. Although mammalian galectin-3 has a weak affinity for Sepharose, sufficient amounts of protein were purified. Purified proteins were stored at 4 °C and used for up to 1 week.

Galectin-3-CRD was prepared as described by Massa et al. (18). Briefly, purified galectin-3 was digested with collagenases VII (Sigma) with a lectin:collagenase ratio of 20:1 by weight in 20 mM Tris- HCl, pH 7.5, 150 mM NaCl, and 2 mM CaCl₂ at 37 °C for 4 h. Adding EDTA to a final concentration of 5 mM stopped the digestion. Then, galectin-3-CRD was purified by asialofetuin-Sepharose affinity chromatography. The use of asialofetuin-Sepharose, instead of unmodified Sepharose, significantly improved the protein yield. Collagenase cleavage was verified by an observed shift in the mobility of galectin-3 in a SDS-PAGE gel.

RESULTS

In a preceding study we applied SCFS to quantify early adhesion of MDCK cells to laminin-111 and collagen-I matrices (13). It was demonstrated that initial adhesion to laminin-111 is integrin-independent, and mediated by carbohydrate interactions. Galactose-binding galectins-3 and -9 facilitate these interactions. In contrast, adhesion to collagen-I was dependent on integrins and the depletion of neither galectin-3 nor -9 had an effect on the early adhesion (20 s contact time).

Prolonged Contact of MDCK Cells to Collagen Substrates, Results in Highly Variable Detachment Forces—Here, we examined the adhesion of control, galectin-3 knockdown (Gal3-KD), and galectin-9 knockdown (Gal9-KD) MDCK cells to collagen-I matrices at contact times of 90 s. The measured detachment forces were highly variable (Fig. 1A) and showed cell-to-cell variations. The forces were not normally distributed, but tailed toward high values (Fig. 1A). Interestingly, the tailing was especially evident in Gal3-KD cells.

![FIGURE 1. The contact area of MDCK cells with the substrate does not correlate with adhesion enhancement. A, normalized histogram of combined detachment forces of 63 cells (295 force curves) after a contact time of 90 s to collagen-I. The data for the three MDCK cell lines (control, Gal3-KD, and Gal9-KD were combined. B, plasma membrane of Gal3-KD cells was fluorescently labeled. Single cells were attached to a ConA-coated AFM cantilever and pressed for 90 s (contact force, 0.75 nN) onto a collagen-IV coated glass coverslip (see also “Experimental Procedures”). The contact area of the cell with the substrate was imaged by time-lapse TIRF microscopy. Images of representative adhesion-enhanced and non-enhanced cells are depicted. The fluorescent signals were outlined to quantify the size of the contact area. C, contact area data points were normalized to the initial area and plotted with respect to time. Scale bar, 1 μm.](JBC_M4_31448_31448_F1)
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characterized this enhanced adhesion state (19). Adhesion-enhancement was attributed to cooperative integrin receptors.

**TIRF Analysis of the Cell-Substrate Contact Area**—The similar distribution of detachment forces on collagen-I supports (Fig. 1A) indicated that the processes leading to adhesion enhancement also occurred in MDCK cells. Inspired by these findings, we characterized adhesive properties of control, Gal3-KD, and Gal9-KD MDCK cells to collagen-I matrices at extended contact times.

Differences in the spreading of cells on the substrate that lead to differences in the contact area could result in deviation in forces needed to detach cells. To determine if adhesion-enhanced cells spread quicker on the substrate, SCFS was combined with TIRF microscopy. Gal3-KD cells were labeled using a fluorescent membrane-intercalating dye. The adhesion to collagen was probed while imaging the contact area. Time-lapse TIRF images were quantified to measure the size of the contact areas (Fig. 1B). The resulting diagrams (Fig. 1C) demonstrated that, although the size of the contact area generally fluctuates more for adhesion-enhanced cells, the enhanced adhesion state was not associated with an increase in the contact area.

**SCFS Analysis of Adhesion to Collagen-I**—Fig. 2A represents the stacked histogram of average detachment forces calculated for individual control, Gal3-KD and Gal9-KD cells. Detachment forces of the three cell lines were combined to increase the number of data points. A cell population distributed around the most probable value (MPV) of 1.3 nN, can be differentiated from a more varied population with a significantly higher MPV of 20 nN (Fig. 2A). Based on the detachment force histogram, a cut-off force of 4 nN was chosen to classify adhesion-enhanced and non-enhanced cells. Representative force-distance curves are depicted in Fig. 2B. Classifying cells according to this criterion allowed the comparison of the detachment forces between the three cell types. The average detachment forces of control and knockdown cells within the two classes revealed no significant difference (Fig. 2C). However, compared with control and Gal9-KD cells, Gal3-KD cells entered the enhanced adhesion state with increased probability (Fig. 2D). This result is in agreement with the distribution of detachment forces (Fig. 1A), where the majority of high detachment forces measured stemmed from Gal3-KD cells.

To analyze by which integrin MDCK cells bind collagen-I, an integrin $\beta_1$-function-blocking antibody (AIIB2) (20) and RGD peptides (21) (competitive inhibitor for RGD-dependent integrins) were used (Fig. 2E). Antibody addition significantly reduced cell adhesion to collagen-I, whereas RGD peptides had no effect, demonstrating that adhesion of MDCK cells to collagen-I is mediated by a $\beta_1$-subunit containing integrin. An isotype-matched rat IgG control antibody did not influence detachment forces, excluding nonspecific effects of AIIB2.

Analyzing the smallest detectable unbinding force units (hereon called force steps; see also Fig. 2B, inset) extracted from force-distance curves, a difference was observed (Fig. 2F; force steps from Gal3-KD cells). Force step sizes from non-enhanced cells were distributed around 94 pN, coinciding with reported
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interaction forces of individual integrins with their ligands (22). Therefore, most of the rupture events from these cells were attributed to the rupture of single integrin-collagen bonds. Similarly, the distribution of force step sizes from adhesion-enhanced cells was centered around an ill-defined peak at 110 pN. However, a broad tail at higher force values marked the distribution, showing that a considerable percentage of rupture events rose above the single molecule force level. This suggests that adhesive units containing cooperating integrin receptors had formed.

**Integrin Clustering, not Integrin Affinity Modulation Causes Adhesion Enhancement**—In a separate experiment control cells were incubated with an antibody, TS2/16, known to increase the affinity of the integrin \( \beta_1 \)-subunits for their ligands (23). Compared with untreated cells, treated cells exhibited significantly higher detachment forces. The percentage of cells that classified as adhesion enhanced (>4 nN detachment force) almost doubled (data not shown). The force step MPV for untreated cells was 93 pN, whereas antibody addition increased the value to 133 pN (Fig. 2G). Both data sets were centered around the MPV but no pronounced tail toward high forces was observed. This shows that there are two distinct mechanisms to increase adhesion; increased integrin affinity, as observed for TS2/16 addition, and increased integrin cooperativity, as postulated in the previous paragraph. Both mechanisms can be distinguished by the size distribution of force steps.

**SCFS Analysis of Adhesion to Collagen-IV**—To test if the increased probability of Gal3-KD cells to enter the enhanced adhesion state is specific to collagen-I, adhesion to collagen-IV was examined. Similar to collagen-I, collagen-IV is recognized by \( \beta_1 \)-subunit containing integrins, evidenced by the significant reduction of MDCK cell adhesion upon AIIB2 addition (Fig. 3A) (24). When probing adhesion of control, Gal3-KD and Gal9-KD cells to collagen-IV, measured detachment forces were highly variable (data not shown). Compared with control and Gal9-KD cells, the probability of adhesion enhancement was again higher for Gal3-KD cells (Fig. 3B). It is concluded that MDCK adhesion to collagen-I and -IV is similar.

**MDCK Cells Do Not Switch to the Enhanced Adhesion State When Adhesion Is Probed to Laminin-332**—MDCK cells express at least four integrins, \( \alpha_2\beta_1, \alpha_3\beta_1, \alpha_6\beta_1 \), and \( \alpha_v\beta_3 \) (25). Adhesion to collagen-I and -IV was likely mediated by integrin \( \alpha_3\beta_1 \), as it is the only collagen-binding integrin identified in MDCK cells (26). It remained to be determined whether galectin-3 only interfered with integrin \( \alpha_3\beta_1 \)-dependent adhesion, or also influenced adhesion by the other \( \beta_1 \)-containing integrin, \( \alpha_6\beta_1 \). Integrin \( \alpha_3\beta_1 \) has been identified as a receptor for laminins (27) and thrombospondin (28). Problematic in the analysis of integrin \( \alpha_3\beta_1 \)-mediated cell adhesion is the lack of ECM proteins, exclusively binding this receptor. For this reason, laminin-332 a ligand to which both, integrin \( \alpha_3\beta_1 \) and \( \alpha_6\beta_1 \) are known to bind was used (29, 30). To separate the contributions of the two integrins to cell adhesion, integrin-blocking antibodies AIIB2 (\( \beta_1 \)-subunit) and GoH3 (\( \alpha_v \)-subunit) were used. Between the three cell types no significant differences in the average detachment force could be detected (Fig. 4A). In accordance with literature, addition of the antibodies AIIB2 or GoH3 reduced, but did not completely blocked adhesion of MDCK cells to laminin-332 (Fig. 4A) (30). Separating force-distance curves into adhesion-enhanced and non-enhanced classes was not possible for the reason illustrated in Fig. 4B. Adhesion to laminin-332 is stronger, with a MPV of 4600 pN, than to collagen-I (960 pN). As the MPV for the adhesion to laminin-332 is higher than the 4 nN cut-off force, used to separate adhesion-enhanced and non-enhanced cells, this criterion could not be applied. Unlike for adhesion to collagen, a high-force tail was not detectable in the force histogram recorded for laminin-332 (Fig. 4B), suggesting the lack of adhesion enhancement. Another characteristic of the enhanced adhesion state was the increased magnitude of force steps (19). Force steps extracted from force-distance curves recorded on laminin-332 and collagen-I were compared (Fig. 4C). Whereas the force step histogram for collagen-I adhesion contains the described high-force tail, the distribution for laminin-332 adhesion lacked this feature. We concluded that adhesion enhancement does not occur when cells were in contact with laminin-332 although a \( \beta_1 \)-subunit-containing integrin was evidently involved.

**SCFS Analysis of Adhesion to Fibronectin**—To analyze if integrin \( \alpha_v\beta_3 \) can induce the adhesion-enhanced phenotype in Gal3-KD cells, fibronectin was used as substrate. Fibronectin, a high molecular weight glycoprotein interacts with several integrins, of which at least the RGD-binding integrin \( \alpha_v\beta_3 \) (28) is expressed in MDCK cells (25). Using AIIB2 and RGD peptides
the specificity of MDCK cell adhesion to fibronectin was probed. As expected, addition of RGD peptides significantly reduced cell adhesion to fibronectin while the addition of AIIB2 and a nonspecific RGE control peptide had no effect (Fig. 5A). Thus, we concluded that the interaction of MDCK cells with fibronectin is mediated by an RGD-binding integrin, possibly \( \alpha_5\beta_1 \).

Compared with collagen-I, the distribution of detachment forces recorded on fibronectin lacked the tail toward high forces (Fig. 5B). An enhanced adhesion state was rarely observed and significant differences in its probability were not detected between the three cell lines (Fig. 5C). This shows that galectin-3 did not influence integrin-mediated adhesion kinetics when cells interacted with fibronectin. In summary, we concluded that galectin-3 acted exclusively through integrin \( \alpha_5\beta_1 \) to inhibit rapid adhesion-enhancement in MDCK cells.

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**FIGURE 4.** Measuring cell adhesion to laminin-332 by SCFS. A, comparison of the average detachment forces between control, Gal3-KD and Gal9-KD cells and control cells in the absence and presence of integrin \( \beta_1 \)-function-blocking antibody AIIB2, integrin \( \alpha_5 \)-function-blocking antibody GoH3 or isotype-matched rat IgG control antibody. Adhesion-enhanced and non-enhanced cells where separated before comparison (control, \( n = 23 \); Gal3-KD, \( n = 20 \); Gal9-KD, \( n = 21 \); control + AIIB2, \( n = 20 \); control + GoH3, \( n = 15 \); control + rat IgG, \( n = 15 \)). B, comparison of the merged detachment forces recorded for control, Gal3-KD and Gal9-KD MDCK cells on collagen-I and laminin-332. C, force steps where extracted from force curves (control, Gal3-KD and Gal9-KD) recorded on collagen-I or laminin-332. The normalized step size probability is plotted. For each substrate force steps from control, Gal3-KD and Gal9-KD were combined; collagen-I, 1783 steps; laminin-332, 1532 steps. The contact time for all experiments was 90 s, unless stated otherwise. *, \( p < 0.05 \); **, \( p < 0.01 \).

**FIGURE 5.** SCFS analysis of cell adhesion to fibronectin. A, average control MDCK cell detachment forces in the absence and presence of integrin \( \beta_1 \)-function-blocking antibody AIIB2, RGD peptides, or nonspecific RGE control peptides (control, \( n = 17 \); control + AIIB2, \( n = 16 \); control + GoH3, \( n = 14 \); control + RGE, \( n = 17 \)). B, comparison of the merged detachment forces for control, Gal3-KD and Gal9-KD MDCK cells recorded on collagen-I and fibronectin. C, percentage of adhesion-enhanced cells when probing adhesion to fibronectin. Error bars in C represent S.E. Contact time: 90 s. ns, \( p > 0.05 \); *, \( p < 0.05 \); **, \( p < 0.01 \).
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A

B

C

D

E

FIGURE 6. Galectin-3 acts at the cell surface to effect integrin α₂β₁-mediated adhesion via galactoside-dependent oligomeric interactions. A, control, MII RCA (mutant cell line lacking galectin-3 ligands at the cell surface; see "Results" section) and Gal3-KD cells were seeded on poly-L-lysine-coated coverslips, fixed and either permeabilized (for detection of total galectin-3) or left untreated (for detection of extracellular galectin-3). Galectin-3 was detected with a monoclonal antibody against the Mac-2 antigen and visualized with a FITC-conjugated secondary antibody. B, comparison of the average detachment forces between control, MII RCA, and Gal3-KD cells. Adhesion-enhanced and non-enhanced cells were separated before comparison (control, n = 15; MII RCA, n = 18; Gal3-KD, n = 24). C, percentage of adhesion-enhanced cells. D, comparison of the average detachment forces between untreated Gal3-KD and Gal3-KD cells preincubated with recombinant, full-length galectin-3 (Gal3) or galectin-3-CRD (Galectin-3-CRD). Adhesion-enhanced and non-enhanced cells were separated before comparison (Gal3-KD, n = 30; Gal3-KD + Gal3, n = 30; Gal3-KD + Gal3-CRD, n = 16). E, percentage of adhesion-enhanced cells. Adhesion was probed to collagen-I, unless stated otherwise. Contact time (B-E): 90 s. Error bars in C and E represent the S.E. ns, p > 0.05; *, p < 0.05; **, p < 0.01.

attempted to show where galectin-3 influences integrin α₂β₁-mediated adhesion. The MDCK mutant cell line MII RCA (32) is defective in translocation of UDP-galactose into the lumen of the Golgi apparatus (33). As a consequence, glycoproteins do not bear galactose-containing side chains, abolishing CRD-mediated interactions of galectin-3 with these proteins (34). In agreement with previous studies, MII RCA cells expressed galectin-3 at levels comparable to wild-type cells, and the protein was not present on the cell surface (Fig. 6A) (34). Thus, MII RCA cells were used to analyze whether galectin-3 influences integrin function on the extra- or intracellular side of the plasma membrane and if galactoside binding is required. When probing adhesion of control, Gal3-KD and MII RCA cells to collagen-I, no differences in overall adhesion strength could be detected, after force curves from adhesion-enhanced and non-enhanced cells had been separated (Fig. 6B). However, compared with control cells the percentage of cells classified as adhesion-enhanced doubled for Gal3-KD and MII RCA cells (Fig. 6C), showing that sugar-mediated interactions at the cell surface are required for the inhibition of adhesion enhancement by galectin-3.

To confirm that galectin-3 acts extracellularly, we rescued the galectin-3 knockdown by addition of exogenous, recombinant galectin-3. Addition of the recombinant lectin significantly reduced detachment forces of non-enhanced Gal3-KD cells from collagen-I (Fig. 6D). Furthermore, the percentage of adhesion-enhanced Gal3-KD cells dramatically decreased upon addition of the recombinant protein (Fig. 6E). In contrast, addition of galectin-3 lacking the N-terminal domain critical for the multivalent behavior of the lectin (35) had no effect on the percentage of adhesion-enhanced Gal3-KD cells (Fig. 6E). This demonstrated that galectin-3 affected integrin α₂β₁ on the extracellular side of the plasma membrane probably by the formation of galectin-glycoprotein lattices.

DISCUSSION

We applied SCFS to analyze the adhesion of MDCK cells to different ECM proteins and determined the contributions of galectins-3 and -9. It was found that galectin-9 did not influence MDCK cell adhesion in any of the conditions probed. Furthermore, differences in adhesive strength or adhesion kinetics were not detected when control, Gal3-KD and Gal9-KD MDCK cells were applied to either fibronectin or laminin-332. In contrast, the adhesion strengths of MDCK cells to collagen-I and -IV showed complex behavior. At a contact time of 90 s, detachment forces varied greatly. In a previous study, these variations were attributed to the switch of a protein to an enhanced adhesion state (19). Galectin-3 Influences Integrin α₂β₁-Mediated Adhesion to Collagen-I and -IV—When probing the adhesion of MDCK cells to collagen-I and -IV, a higher percentage of Gal3-KD cells entered the enhanced adhesion state. By applying a combined
TIRF/AFM setup to analyze the contact area between cells and their substrate during SCFS experiments we demonstrated that cell spreading was not associated with adhesion enhancement. Using an integrin β1-function-blocking antibody, we showed that adhesion to collagen-I and -IV was mediated by a β1 subunit containing integrin α2β1. A Gal3-KD-dependent increase in the percentage of adhesion-enhanced cells was not observed when fibronectin or laminin-332 were probed. The interaction of MDCK cells with these proteins was mediated by integrins other than integrin α2β1. Therefore, it was concluded that galectin-3 specifically influences integrin α2β1-mediated adhesion. The inhibitory effect of galectin-3 on adhesion kinetics was duplicated by the addition of recombinant galectin-3 to Gal3-KD cells. Furthermore, a mutant MDCK cell line deficient in extracellular ligands for galectin-3 showed the same stimulatory phenotype as observed in galectin-3-depleted cells. This demonstrated that galectin-3 acts at the cell surface presumably by binding to glycoproteins via its lectin-domain. This is consistent with earlier in vivo studies showing that galectin-3 associated with cell surface glycoproteins and glycosylated components of the ECM (8).

It should be noted, that the adhesion promoting effect of galectin-3 depletion holds true only for the relatively short contact times applied in this study. When adhesion to collagen-I was probed for longer contact times (90 min) using traditional adhesion assays, both Gal3- and Gal9-KD cells exhibited adhesion defects (13).

Galectin-mediated Integrin Affinity Modulation Is Not the Cause for Adhesion Enhancement—To explain the impact of galectins on cell adhesion, two modes of action have been proposed (12). First, galectins directly promote the binding of cells to substrates by cross-linking appropriately glycosylated receptors on opposing surfaces (13). Secondly, galectins indirectly affect cell adhesion by binding to extracellular domains of transmembrane glycoproteins, thereby modulating the binding affinity, clustering or availability (endocytosis) of these proteins to their extracellular ligands (11, 36, 37). Unlike for the laminin-111-galectin interactions reported in our previous study (13), the direct mode of action cannot account for the results obtained in this study. The addition of integrin function-blocking reagents inhibited the adhesion of MDCK cells to all substrates tested. If galectin-3 is directly involved in adhesion, its contribution is small and cannot be separated from nonspecific interactions. More likely is that galectin-3 indirectly influences MDCK cell adhesion by interacting with integrin receptors, thereby modulating the affinity or avidity of the integrins for their ligands. When comparing the force step histograms of adhesion-enhanced and non-adhesion-enhanced cells, the positions of their main peaks concurred (Fig. 2F), while the number of high force steps differed. Therefore, it was concluded that the avidity but not affinity of the integrin receptors changed in the enhanced adhesion state.

Multivalency Is Important for Galectin Function—Almost all galectins either contain two CRDs or dimerize. Bivalence is a prerequisite for their modulation of cell-cell and cell-pathogen interactions and possibly allows them to form lattices by ligand-cross-linking. Galectin-3, the sole chimeric galectin does not form dimers in solution (18, 38). However, upon oligosaccharide binding, galectin-3 forms oligomers via their N-terminal domains (10, 35). Galectin-3 lattices are proposed to be involved in receptor trafficking and receptor clustering. Partridge et al. (39) showed that galectin-3 lattices retained epidermal growth factor and transforming growth factor-β receptors on the cell surface by interfering with their endocytosis. On the surface of T-cells, galectin-3 lattices restricted the recruitment of T-cell receptors to the site of antigen presentation (11). Similarly, galectin-3 regulated the dynamics of fibronectin fibrillogenesis as well as associated integrin activation and translocation via receptor cross-linking (40). Galectin-3 lattices were found to be robust and resist lateral diffusion (9), reinforcing findings that these lattices regulate the lateral movement and recycling of cell surface receptors. A similar mechanism is supported by our results. If galectin-3 binds N-glycans on integrin α3β1, the receptor might be sequestered within a multivalent galectin-glycoprotein lattice, thereby impeding receptor clustering. Consequently, the kinetics of integrin-mediated adhesion would be slowed. In Gal3-KD cells, the absence of galectin-3 may increase the mobility of the integrin. Receptor mobility has been correlated with enhanced cell adhesion and adhesion strengthening (41, 42).

Integrin clustering is often associated with the observation of macroscopic adhesion patches, such as focal complexes and adhesions. These structures can be typically detected 10–20 min after cell seeding. However, functional integrin adhesion complexes may first form as small receptor clusters that cannot be resolved by conventional light microscopy (43). Indeed, SCFS appeared to detect the establishment of cooperative integrin binding within 90 s of matrix contact, suggesting that integrins start clustering before this becomes optically detectable.

Integrins α2β1, α3β1, α5β1, and αβ4 are clearly involved in the adhesion of MDCK cells to the ECM proteins tested. However, it cannot be excluded that MDCK cells express other integrins. Transcriptional profiling suggested that MDCK cells also have transcripts for the α5β1, β5, and β6 integrin subunits.3 If expressed, these integrin subunits would form functional integrin receptors. However, integrin α6β4, along with α3β1 and αβ5, two fibronectin receptors that could combine from the described integrin subunits, where not found in MDCK cells (25). Importantly, addition of an integrin β1-function-blocking antibody (AIIB2) to MDCK cells did not decrease adhesion to fibronectin, suggesting that no β1-subunit containing integrin was involved (Fig. 2B). According to the transcription profiles, the integrin α5-subunit is not expressed in MDCK cells, excluding an important collagen-binding integrin, α3β1, as a potential binding partner for galectin-3. Two collagen-binding integrins α10β1 and α11β1 were recently identified. The distribution of both receptors is restricted. Integrin α10β1 is mainly expressed in chondrocytes (44), whereas α11β1 is mainly found in the mesenchyme (45). As neither receptor has been identified in epithelial cell lines, it was concluded with some certainty that these integrins do not participate in MDCK cell adhesion to either collagen-I or -IV.

3 A. Manninen, unpublished data.
Galectin-3 Influences Integrin $\alpha_2\beta_1$ Cooperativity

In summary, our study showed that depletion of galectin-3 in MDCK cells led to an increased probability for the enhanced adhesion state when adhesion was probed to collagen-I or -IV. The enhanced adhesion state was characterized by heightened detachment forces and by an increase in the force steps to values above those expected for the breakage of single integrin-ligand bonds, suggesting cooperative integrin receptors. The effect of galectin-3 depletion was specific to integrin $\alpha_2\beta_1$-mediated adhesion and not observed when adhesion was mediated by other integrins.

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