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Characterization of the interaction between hydroxypropyl guar galactomannan and galectin-3

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ABSTRACT

Multivalent galactose ligands have been proposed for selective targeting of carbohydrate-binding proteins on epithelial cell surfaces, both in normal and pathological conditions. One cellular partner is galectin-3, a β-galactoside-binding protein present on many epithelial linings, such as those of the ocular surface. In this study, we investigated the ability of hydroxypropyl guar galactomannan (HPGG) to bind recombinant galectin-3 and to target the apical surface of differentiated human corneal keratinocytes. Pull-down and slot-blot assays demonstrated that fluorescence-labeled HPGG bound recombinant galectin-3 through a galactose-dependent mechanism. In contrast, no binding of HPGG could be detected towards recombinant galectin-8 or -9. In a cell culture system, HPGG bound weakly to biotinylated cell surface corneal isolates containing endogenous galectin-3, and incubation of HPGG with corneal keratinocytes in culture resulted in discrete, galactose-independent, binding to the cell surface. Moreover, HPGG failed to elute the biological counter-receptor MUC16 from galectin-3 affinity columns. We conclude that HPGG binds galectin-3 through the conventional carbohydrate-recognition domain *in vitro*, but not in a biological system, suggesting that endogenous carbohydrate ligands on epithelial cell surface glycocalyces impair HPGG biorecognition.

1. Introduction

Polysaccharides are the most abundant and most diverse biopolymers in nature, where they participate in many crucial biological processes due to their specific, carbohydrate-mediated interactions with physiological receptors on plasma membranes [1]. These molecules, as well as synthetic polymeric derivatives carrying saccharide side chains, have been proposed for diverse therapeutical carbohydrate-based applications involving specific recognition of cell surfaces, such as vaccination, inhibition of pathogenic binding, and drug delivery [2–4].

Guar galactomannan is a high molecular weight (approximately 50–8000 kDa) polysaccharide obtained from the seeds of *Cyamopsis tetragonolobus* (L.) Taub. It contains a linear backbone of β1–4 linked mannose units with α1–6 galactose residues occurring as side branches. During the last decade, guar galactomannan and its synthetic derivatives have been shown to provide therapeutic benefits in a wide spectrum of biomedical applications, such as gastroenterologic disease, targeted drug delivery in cancer, and dry eye, a common ocular disease affecting millions of people around the world [5–7].

Cell surface lectins play important roles in a variety of biological processes, including intercellular recognition, cell adhesion, cell growth, and cell differentiation [8]. Galectins are a family of animal lectins defined by their evolutionarily conserved carbohydrate recognition domain (CRD) and affinity for β-galactoside-containing oligosaccharides [9]. Members of this family have been detected in many cell and tissue types, and their overexpression is associated with neoplastic progression [10]. The membranous location of galectins on cell surfaces has prompted research into galectins as a potential target for carbohydrate-based drug delivery vehicles [3,10–12]. More recently, it has been shown that human recombinant galectin-1 binds Davanat, a galactomannan polymer presently being used against metastatic colorectal cancer [13]. Galectin-3 is a 35 kDa protein highly expressed on human ocular surface epithelia. Like other galectins, galectin-3 can interact in a multivalent fashion and cross-link glycan ligands to generate molecular lattices on cell surfaces [14–16]. In this study, we used pull-down and

Abbreviations: CRD, carbohydrate recognition domain; DMEM/F12, Dulbecco's modified Eagle's medium/F12; FLA, fluoresceinamine; HPGG, hydroxypropyl guar galactomannan; PBS, phosphate-buffered saline; PNA, peanut agglutinin; rGal, recombinant galectin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, tris-buffered saline; TRED, Texas red; WGA, wheat germ agglutinin.

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affinity assays to investigate whether hydroxypropyl guar galactomannan binds galectin-3 *in vitro*, and in a biological system of human corneal keratinocytes expressing endogenous galectin-3.

2. Materials and methods

2.1. Preparation of fluorescence-labeled HPGG

Experiments were performed with fluoresceinamine (FLA)-conjugated HPGG or Texas red (TRED)-conjugated HPGG. Synthesis of FLA-HPGG was based on a modification of the technique developed by Arnosti et al. [17]. Briefly, a mixture containing 3 mg each of cyanogen bromide and HPGG in water was maintained at pH 11 for 7 min by addition of 0.25 M NaOH. The activated polysaccharide was allowed to precipitate in 100% ethanol for 5 min on an end-over-end rocker. After centrifugation, the pellet was washed three times in ethanol, lyophilized, and resuspended in 5 ml of 150 mM $Na_2B_4O_7$ containing 2 mg FLA (Sigma Aldrich; St. Louis, MO). The mixture was allowed to react for 18 h in the dark at room temperature, followed by precipitation of the conjugated polymer in ethanol and resuspension in phosphate-buffered saline (PBS), pH 8. The labeling efficiency was determined using the phenol sulfuric acid method [18], for carbohydrate concentration, and fluorometry (Ex485/Em535; Tecan Genios; Maennedorf, Switzerland), for FLA concentration, using galactose and FLA standards, respectively. Labeling densities of at least 50 (μM FLA/μM HPGG) were used in pull-down and cell culture affinity experiments. TRED-HPGG was obtained from Alcon Research, Ltd. (Fort Worth, TX).

2.2. Cell culture

Telomerase-immortalized human corneal limbal epithelial (HCLE) cells were cultured in a medium optimized for proliferation of keratinocytes (keratinocyte serum-free medium; Invitrogen Corp.; Carlsbad, CA) to achieve confluence, as previously reported [19]. After reaching confluence, cell cultures were grown in Dulbecco's modified Eagle's medium/F-12 (DMEM/F12 Sigma–Aldrich) supplemented with 10% calf serum (Hyclone; Pittsburgh, PA) and 10 ng/ml epidermal growth factor (Invitrogen) for 7 days to promote stratification and differentiation [19].

2.3. Biotinylation of cell surface proteins

Apical cell surface proteins from stratified cultures of HCLE cells were biotinylated and isolated on NeutrAvidin™-agarose beads using the Pinpoint™ Cell Surface Protein Isolation kit (Thermo Scientific; Rockford, IL) according to the manufacturer's instructions. Beads were then used in pull-down assays as described below. An aliquot (25 μl) of biotinylated protein was diluted in Laemmli buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and analyzed for the presence of endogenous galectin-3 by western blot using undiluted rat monoclonal M3/38 antibody.

2.4. Pull-down assays

50 microliter of agarose beads bound to peanut agglutinin (PNA, Sigma Aldrich), wheat germ agglutinin (WGA, Sigma Aldrich), and recombinant galectin-3 (rGal-3) were gently shaken with 300 μl of 0.1 mg/ml FLA-HPGG for 1 h at room temperature in the dark. Beads were centrifuged, washed 3 times with PBS, and dried on glass slides. Beads were covered with coverslips containing Vectashield mounting medium (Vector Labs; Burlingame, CA) and imaged using an Eclipse E800 fluorescence microscope (Nikon Inc.; Melville, NY). Experiments were performed in triplicate, with 20 images taken per condition in each experiment. Mean intensity per bead was determined using Adobe Photoshop CS software (San Jose, CA). For competitive inhibition studies, galectin-3 beads were incubated with 200 μl of 0.5 mg/ml FLA-HPGG in the presence of 0.1 M lactose (Galβ1–4Glc) or sucrose (Fruα2–1Glc). For cell surface experiments, NeutrAvidin™-agarose beads (Thermo Scientific) containing biotinylated cell surface proteins or beads alone were incubated with 500 μl of 2.5 mg/ml TRED-HPGG as described above. Galectin-3 conjugated beads incubated with TRED-HPGG were used as positive control.

2.5. Slot-blot assay

50 microliter serial dilutions of HCLE cell lysates, HPGG, mannan (Manβ1–4Man), and glycogen (Glcα1–4Glc) were applied individually to a nitrocellulose membrane in a slot-blot apparatus (Bio-Rad; Hercules, CA). Nitrocellulose membranes were then removed and blocked with 1% polyvinylpyrolidine in tris-buffered saline (TBS), pH 7.5, for 45 min. The membranes were then overlaid for 45 min with 500 ng rGal-3 in 10 ml TBS, washed thoroughly, and probed with undiluted rat monoclonal M3/38 antibody to galectin-3 for 30 min. After washes with TBS, membranes were incubated with goat anti-rat IgG (1:80,000; Santa Cruz Biotechnology; Santa Cruz, CA) for another 30 min. For incubations with rGal-8 and rGal-9, nitrocellulose membranes were blocked for 45 min with 3% fish gelatin (Sigma Aldrich) in PBS, pH 7.5, and incubated for 45 min with 500 ng of rGal-8 and -9 (Abnova; Taipei, Taiwan) in 10 ml TBS. After extensive washing, membranes were probed with goat polyclonal D-18 antibody to galectin-8 (1:1000; Santa Cruz Biotechnology), or goat polyclonal C-20 antibody to galectin-9 (1:1000; Santa Cruz Biotechnology) for 30 min, followed by incubation with donkey anti-goat IgG (1:5000; Santa Cruz Biotechnology) for another 30 min. Antibody binding was detected by development with West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's instructions. Densitometry was performed using NIH Image software (ImageJ; Bethesda, MD).

2.6. In vitro polymer binding and fluorometry

HCLE cells were grown to stratification in Lab-Tek 2-well cell culture slides (Nalge Nunc International; Naperville, IL). The culture medium was replaced with DMEM/F12 medium containing 2.5 mg/ml FLA-HPGG followed by incubation for 1 h at 4 °C to prevent internalization [20]. Unbound polymer was removed by washing with DMEM/F12. Cell culture slides were covered with coverslips containing Vectashield mounting medium, then, imaged by fluorescence microscopy. For fluorometry, HCLE cells grown in 96-well plates (Black/clear bottom; Corning Inc.; Corning, NY) were incubated with 2.5 mg/ml TRED-HPGG alone, or in the presence of 0.1 M lactose or sucrose, for 1 h at 4 °C. Binding was assessed by microplate fluorometry (Ex485/Em535) using a Tecan Genios fluorometer. Experiments were performed in quadruplicate; 12 wells were analyzed for each condition. The autofluorescence of empty wells was subtracted from each reading prior to analysis.

2.7. MUC16 competitive elution assay

A 0.1 ml bed of galectin-3 conjugated beads was mixed with 250 μg of HCLE cell protein lysate in radio immunoprecipitation assay buffer plus protease inhibitors at room temperature for 1 h. After the beads were washed with PBS, the bed was sequentially incubated for 5 min with 0.5 ml each of 0.1 M sucrose, 0.15% HPGG, 0.2% HPGG, 0.25% HPGG and 0.1 M lactose. Aliquots (25 μl) of proteins eluted in the supernatant were analyzed by SDS–PAGE,

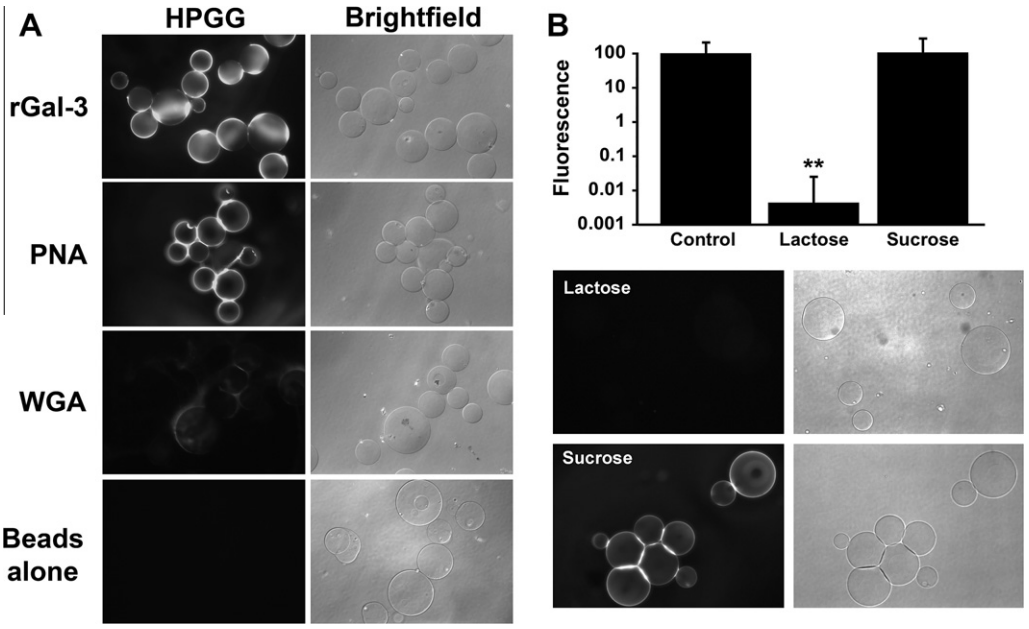

Fig. 1. Galactose-recognizing lectins bind the galactomannan derivative HPGG. (A) FLA-HPGG was incubated with lectin-conjugated agarose beads or beads alone for 1 h at room temperature in the dark. Beads were then centrifuged and applied to a glass slide for fluorescence imaging. Fluorescence micrographs show intense binding of HPGG to rGal-3 and PNA, but not to WGA. Brightfield images in the right panel indicate the position of the agarose beads. Images were obtained using a 20X objective lens. (B) HPGG binds rGal-3 through a galactose-dependent mechanism. Pull-down experiments were performed with galectin-3 agarose beads incubated with FLA-HPGG in the presence of lactose, a competitive carbohydrate inhibitor of galectin binding, or sucrose, a disaccharide control. Images were obtained using a 20X objective lens and analyzed using Adobe Photoshop CS software. Incubation of galectin-3 beads with lactose resulted in abrogation of HPGG binding as compared to sucrose. Representative images demonstrating abrogation of HPGG binding to galectin-3 beads are shown in the bottom panel. Fluorescence measurements are given as mean intensity per bead and are normalized to control. Error bars represent the standard error of the mean (***p<0.001, Tukey–Kramer multiple comparisons test).

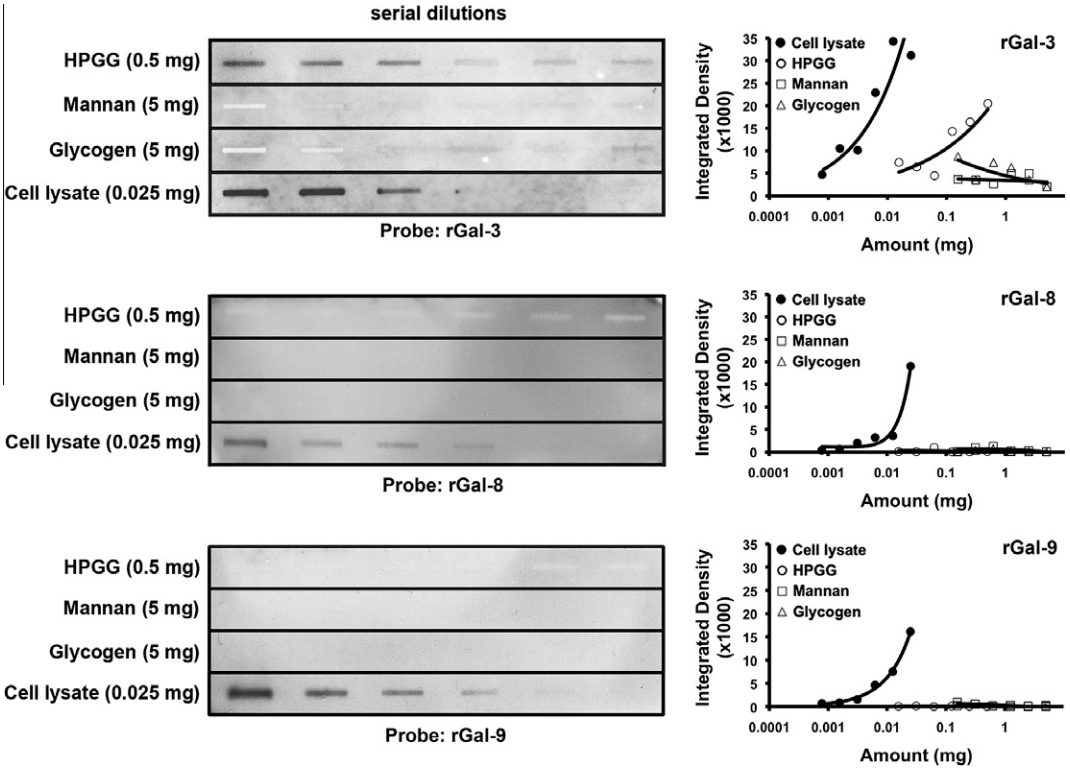

Fig. 2. Slot-blot filter assay proves interaction of rGal-3 with HPGG. Two-fold dilutions of HPGG, mannan and glycogen were vacuum blotted onto nitrocellulose membranes, probed with rGal-3 and overlaid with the M3/38 antibody. rGal-3 bound to HPGG but not to mannan or glycogen. By densitometry, binding of rGal-3 to HPGG could be detected in amounts as low as 0.125 mg. No binding of rGal-3 could be detected at concentrations of up to 5 mg of mannan and glycogen. Moreover, rGal-8 and -9 failed to bind HPGG, mannan, and glycogen at the concentrations used in this study. Cell lysates from cultures of human corneal keratinocytes expressing endogenous galectin-3, -8 and -9 were used as positive controls.

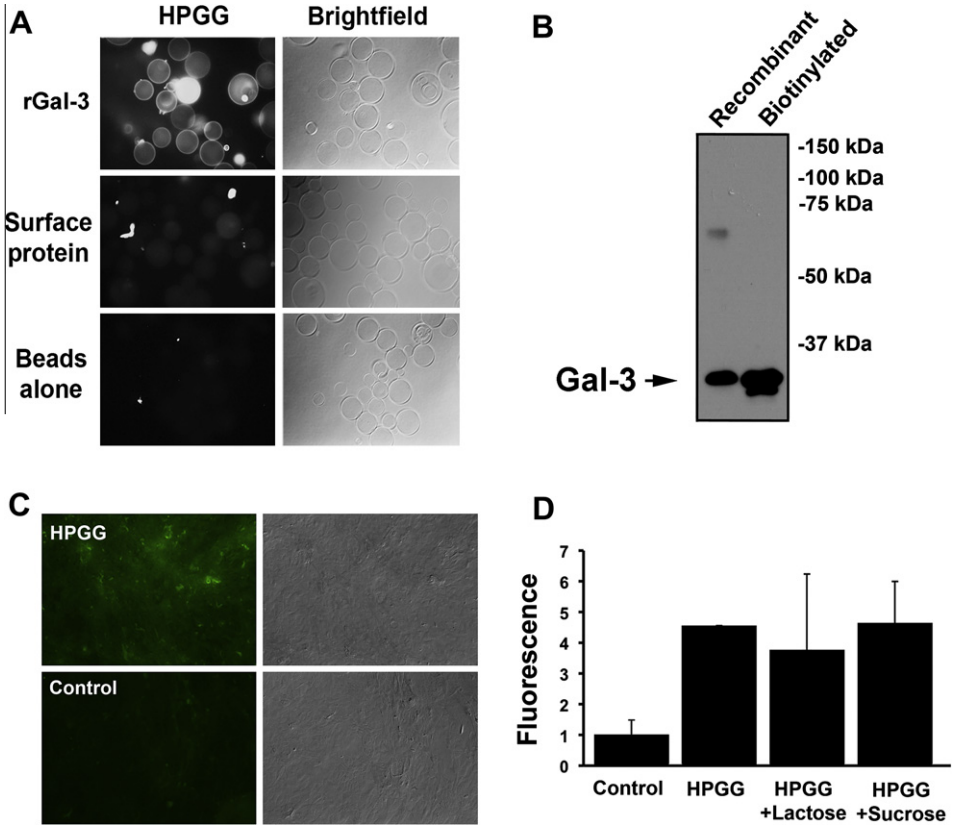

Fig. 3. The interaction between HPGG and components of the cell surface glycocalyx is impaired in human corneal keratinocytes expressing endogenous galectin-3. (A) Weak binding of TRED-HPGG is observed to biotinylated cell surface protein immobilized on affinity columns as compared to that observed in rGal-3-conjugated beads. Brightfield images in the right panel indicate the position of the beads. (B) The presence of endogenous galectin-3 in biotinylated cell surface isolates was confirmed by western blot. rGal-3 (50 ng) was used as positive control. (C) As determined by fluorescence microscopy, incubation of FLA-HPGG with stratified human keratinocytes for 1 h at 4 °C resulted in discrete binding of the polysaccharide to punctated areas on the apical cell surface (arrows). No polymer was added in the control condition. Corresponding brightfield images are shown to the right. (D) By fluorometry, no effect was observed when incubating epithelial cells with TRED-HPGG in the presence of 0.1 M lactose or sucrose as compared to polymer alone. Error bars represent the standard error of the mean.

followed by western blot using the M11 antibody against MUC16 (1:3000; Neomarkers; Fremont, CA) as described [21].

3. Results

3.1. HPGG binds rGal-3 through a galactose-dependent mechanism

To determine whether HPGG can interact with recombinant human galectin-3 in a galactose-dependent manner, we performed pull-down assays by incubating fluorescence-labeled HPGG with galectin-3 immobilized on agarose beads. As determined by fluorescence microscopy, HPGG bound to the surface of galectin-3 beads (Fig. 1A). Similar surface interactions were detected when HPGG was incubated with beads coated with galactose-binding PNA, but not with mannose/glucose-binding WGA, indicating that the galactose side branches on the HPGG polymer are accessible to galactose-specific lectins. Competitive inhibition experiments were performed to confirm the specificity of the interaction between HPGG and rGal-3. In these experiments, incubation of HPGG with galectin-3 beads in the presence of β-lactose, a competitive carbohydrate inhibitor of galectin binding, resulted in abrogation of HPGG binding compared to sucrose, a control disaccharide (Fig. 1B).

The affinity of HPGG for rGal-3 was further assessed in slot-blot assays using two-fold dilutions of the polysaccharide immobilized on nitrocellulose membranes. As shown in Fig. 2, binding of rGal-3 to HPGG was detected in amounts as low as 125 μg. On the other hand, no binding of rGal-3 to mannan and glycogen could be detected at concentrations of up to 5 mg of polysaccharide. Interestingly, no binding was detected between HPGG and rGal-8 and -9, two galectins also expressed by ocular surface keratinocytes [22], at the concentrations used in this study (Fig. 2). In these experiments, cell lysates from cultures of human corneal keratinocytes containing endogenous galectin-3, -8, and -9 were used as positive controls.

3.2. HPGG interaction with endogenous galectin-3 is impaired in human corneal keratinocytes

Given that HPGG binds rGal-3 *in vitro*, we next determined whether HPGG could bind to cell surfaces containing endogenous galectin-3 in a biological system. Surface membrane proteins from human corneal keratinocytes were subjected to biotinylation, then affinity-captured and purified on neutravidin beads, and finally analyzed for interaction with HPGG. As shown in Fig. 3A, beads conjugated to cell surface proteins bound weakly to HPGG, and displayed significantly lower fluorescence intensity as compared to beads coated with rGal-3. The presence of endogenous galectin-3 in the biotinylated cell surface material was confirmed by western blot (Fig. 3B).

The interaction between HPGG and the apical cell surface components was further evaluated by fluorescence microscopy and fluorometry in stratified cultures of human corneal keratinocytes. For these experiments, incubations were performed at 4 °C to prevent internalization of the polymer [20]. By fluorescence

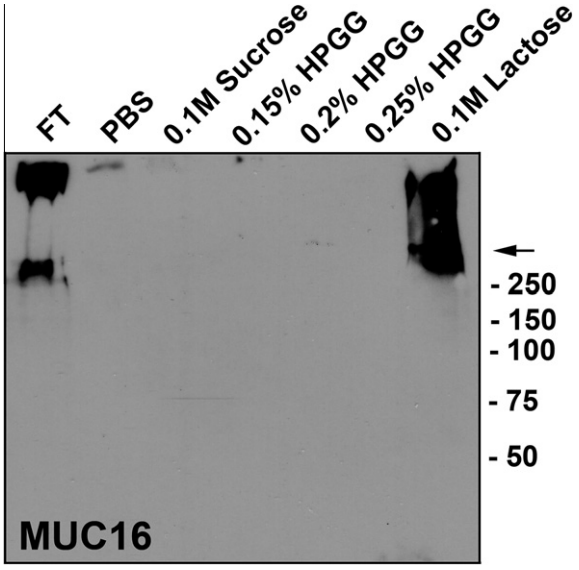

Fig. 4. HPGG fails to elute the biological counter-receptor MUC16 from galectin-3 affinity columns in competitive binding assays. Pull-down experiments were performed by incubation of galectin-3 beads with corneal cell lysates containing endogenous MUC16. After washing with PBS, beads were incubated consecutively with sucrose, increasing concentrations of HPGG, and lactose. MUC16 in the fractions was detected by western blot using the M11 antibody. Incubation of the beads with sucrose and HPGG failed to elute MUC16 from galectin-3 affinity columns. Elution of MUC16 occurred only after addition of 0.1 M lactose to the galectin-3 affinity column. FT, unbound MUC16 after initial incubation of galectin-3 beads with corneal cell lysates. The arrow indicates the interface between stacking and running gel.

microscopy, HPGG bound to stratified cells in a discrete, punctated pattern (Fig. 3C). Moreover, addition of lactose to the cell culture media did not affect binding in fluorometry experiments (Fig. 3D), indicating that the interactions between HPGG and the apical cell surface glycocalyx in human corneal keratinocytes are not galactose dependent.

3.3. HPGG fails to elute the biological counter-receptor MUC16 from galectin-3 affinity columns

Several binding partners have been identified for endogenous galectin-3 on cell surfaces. The membrane-tethered mucin MUC16 is a major counter-receptor on the apical surface of human corneal keratinocytes [16]. We performed a competitive elution assay to determine whether HPGG could compete with MUC16 in binding affinity towards galectin-3. As shown in Fig. 4, incubation of the beads with sucrose and increasing concentration of HPGG (0.15–0.25%) failed to elute MUC16 from galectin-3 affinity columns. As determined by western blot, elution of MUC16 occurred only after addition of 0.1 M lactose to the galectin-3 affinity columns.

4. Discussion

Guar galactomannan is a non-ionic, hydrophilic polysaccharide found abundantly in nature with many properties desirable for biomedical applications. Special attention has been given to galactomannan and its derivatives as colon-specific, oral, and transdermal drug delivery systems [23]. It has been proposed that the bioactivity of multivalent galactoside ligands in recognition processes is driven through preferential association with cell surface receptors [10,12,13]. Here, we report that hydroxypropyl guar galactomannan acts as a ligand for galectin-3 *in vitro*, but does not bind to cultures of human corneal keratinocytes expressing endogenous galectin-3, most likely due to the presence of endogenous carbohydrate ligands on the epithelial glycocalyx that impair polymer biorecognition.

Previous reports have demonstrated the interaction between galactose-containing polysaccharides and recombinant forms of human galectins [11,13,24,25]. Using nuclear magnetic resonance spectroscopy, Miller et al. showed that galectin-1 binds Davanat, a 59 kDa molecular weight galactomannan being used against metastatic colorectal cancer [13]. Interestingly, binding of Davanat was observed over a large area on the surface of galectin-1 that traverses the dimer interface on a site opposite to the CRD, and was dependent on the mannan backbone. In these experiments, the CRD in the galectin-1/Davanat complex remained accessible to other interactions, as lactose could still bind with no apparent loss of affinity [13]. In contrast, our experiments indicate that galactose residues on galactomannan, and the CRD on galectin-3, are major contributors to the interaction. First, HPGG bound strongly with beads coated with galactose-binding PNA, but not with mannose/glucose-binding WGA. Second, HPGG bound recombinant human galectin-3 in pull-down assays, and the interaction was abrogated by lactose. Third, HPGG but not mannan bound rGal-3 in slot-blot assays. The fact that galectin-1 and -3 bind galactomannan through different mechanisms is not entirely surprising, as studies with microarrays containing glycans obtained from natural sources show substantial differences in carbohydrate specificity between the two lectins [26]. Additionally, binding of galactomannan to different galectins could also be influenced by the concentration of the lectin and mode of presentation of the carbohydrate in the different assays employed [27,28]. Experiments in Fig. 2 showing that HPGG binds galectin-3 but not galectin-8 and -9 further supports the concept that individual galectins interact through different mechanisms with exogenous biopolymers, suggesting that galectins have unique physiological roles on epithelial cell surfaces.

Targeting galectins expressed on epithelial cell surfaces (e.g., ocular surface) with galactose-containing polymers has been proposed for targeted delivery of drugs and modulation of a broad spectrum of biological responses [10,11,29–34]. By clustering multivalent galactoside ligands into methacrylate-based polymers, David et al. demonstrated enhanced, lactose-dependent, biorecognition of the polymer in human hepatocarcinoma HepG2 cells [3]. The higher the saccharide moiety content in the methacrylate polymers, the higher the levels of biorecognition. *In vivo* experiments further demonstrated that intravenous administration of methacrylate-based polymers with high galactosamine content to rats results in accumulation of the polymer on the hepatocyte plasma membrane [35]. Multivalent galactoside ligands on methacrylate polymers have also been found to facilitate biorecognition of the anticancer drug doxorubicin by Colo-205, SW-480 and SW-620 cancer cell lines expressing galectin-3 RNA and protein [12]. However, in these experiments, Colo-205 cells did not express a detectable amount of cell surface galectin-3, suggesting that recognition of the galactose-bearing copolymer was not mediated solely by galectin-3. Our data using human corneal keratinocytes indicate that galactomannan binds weakly to both purified plasma membrane protein and discretely to apical surfaces of stratified human keratinocytes expressing galectin-3 on the plasma membrane. Moreover, competitive inhibition assays with lactose did not significantly alter the interaction between HPGG and apical cells in culture, suggesting that galectin-3, or additional galactoside-binding proteins on the epithelial cell surface, binds endogenous ligands and not HPGG on the corneal epithelial glycocalyx. The finding that HPGG fails to elute the biological counter-receptor MUC16 from galectin-3 affinity columns further supports this hypothesis.

In summary, we conclude that the galactomannan derivative HPGG binds galectin-3 through the conventional carbohydrate-binding domain *in vitro*, but not in a biological system, and provide

evidence indicating that the presence of endogenous carbohydrate ligands impairs HPGG biorecognition on the corneal epithelial glycocalyx.

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