Strikingly Different Localization of Galectin-3 and Galectin-4 in Human Colon Adenocarcinoma T84 Cells

GALECTIN-4 IS LOCALIZED AT SITES OF CELL ADHESION

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Two β-galactoside-binding proteins were found to be prominently expressed in the human colon adenocarcinoma T84 cell line. Cloning and sequencing of one, a 36-kDa protein, identified it as the human homolog of galectin-4, a protein containing two carbohydrate binding domains and previously found only in the epithelial cells of the rat and porcine alimentary tract. The other, a 29-kDa protein, is galectin-3, containing a single carbohydrate binding domain, previously found in a number of different cell types including human intestinal epithelium. Despite the marked similarities in the carbohydrate binding domains of these two galectins, their cellular distribution patterns are strikingly different and vary with cellular conditions. In confluent T84 cells, galectin-4 is mostly cytosolic and concentrated at the basal membrane, whereas galectin-3 tends to be concentrated in large granular inclusions mostly at the apical membrane. In subconfluent T84 cells, each galectin is distributed to specific domains of lamellipodia, with galectin-4 concentrated in the leading edge and galectin-3 more proximally. Such different localization of galectins-4 and -3 within T84 cells implies different targeting mechanisms, ligands, and functions. The localization of galectin-4 suggests a role in cell adhesion which is also supported by the ability of immobilized recombinant galectin-4 to stimulate adhesion of T84 cells.

Gaelectins are a family of animal lectins defined by affinity for β-galactoside-containing saccharides and by shared sequence elements (for review, see Ref. 1). Previous work has shown that galectin-4 and galectin-3 are both present at high concentration in intestinal extracts (2–4). Galectin-4 has only been found in the epithelium of the alimentary tract, including oral mucosa (5), esophagus (6), and intestinal mucosa (3), whereas galectin-3 is also abundant in other cells, especially macrophages (7, 8).

Although galectins are cytosolic proteins lacking signal sequences, galectins-1 and -3 are known to be externalized by nonclassical secretory mechanisms (9–12), suggesting that otherers, including galectin-4, might also be released from cells in this way. Galectin-1 and galectin-3 have been shown to bind extracellular matrix components (13, 14) and modulate cell adhesion (15, 16). Hence, it is plausible that galectin-4 is also involved in cell adhesion by interacting with extracellular glycoconjugates. No specific ligands for galectin-4 have been identified so far, but its interaction with an adherens junction component in oral mucosa has been indicated (5).

As a step in determining the function of galectin-4, we have examined its localization in the human colonic adenocarcinoma T84 cell line frequently used as a model of intestinal crypt epithelium (17–19) and compared it with the localization of galectin-3. We found that despite the marked similarities in the carbohydrate binding domains of these two galectins, their distribution patterns in the same cells are strikingly different, with galectin-4 localized mainly at sites of cell adhesion.

EXPERIMENTAL PROCEDURES

Materials—An [35S]protein-labeling mixEXPRESS®S (>1,000 Ci/mmol) was purchased from DuPont NEN. Sepharose CL-2B was obtained from Pharmacia Biotech Inc., and protein G-Sepharose was obtained from Zymed (South San Francisco, CA). The peroxidase substrate kit was from BioGenex (San Ramon, CA). All other reagents, unless specified, were obtained from Sigma.

Antibodies—A rat monoclonal anti-galectin-3 (anti-Mac-2) (7, 8) was used as described (20). Rabbit anti-galectin-4 serum was raised against the N-terminal domain of rat intestinal galectin-4 as described (3). A monoclonal anti-cytokeratin antibody 7D3 was a generous gift from Dr. Caroline Dansky, University of California, San Francisco (21). A mouse monoclonal antibody (IgG2a) against the intracellular domain of human E-cadherin was purchased from Transduction Laboratories (Lexington, KY). Fluorescein-conjugated rabbit anti-rat antibodies, biotinylated sheep anti-rat antibodies, streptavidin-conjugated fluorescein, and streptavidin-conjugated Texas Red were purchased from Amersham Corp. Biotinylated goat anti-rabbit antibodies, Vectastain a, and Vectashield were from Vector (South San Francisco, CA).

Cell Culture—T84 cells (passages 54–70) were grown following the procedure of Dharmsathaphorn et al. (22), in Dulbecco’s modified Eagle’s H-16/F-12 medium (1:1) containing 5% newborn calf serum, 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 15 mM HEPES, pH 7.3, 17.5 mM glucose, and 2.5 mM glutamine, in a humidified atmosphere of 95% air and 5% CO2 at 37 °C (seeding density of 2.5 × 104 cells/cm2). For immunocytochemistry, the cells were grown on uncoated glass coverslips.

Metabolic Labeling and Cell Lysis—For labeling in vivo with [35S]methionine/cysteine, T84 cells were grown on 10-cm-diameter plastic dishes as described above. Endogenous methionine/cysteine was depleted before labeling by washing cells with methionine-free minimal essential medium and incubating in depletion medium (methionine-free minimal essential medium, 5% dialyzed fetal calf serum, 10 mM HEPES, pH 7.3) for 1 h at 37 °C. For labeling, the medium was replaced with 5 ml of the same medium containing 1 mCi of [35S]methionine/cysteine. After 12 h at 37 °C the cells were washed extensively, first in

14294 This paper is available on line at http://www-jbc.stanford.edu/jbc/
prewarmed, then in ice-cold PBS (1.4 mM sodium/potassium phosphate, 135 mM NaCl, pH 7.4) and lysed in 1.5 ml plate of ice-cold lysis buffer: 1 mM EGTA, 1.5 mM MgCl2, 5 mM β-mercaptoethanol, 2.5% Triton X-100 (v/v) in PBS, containing protease inhibitors (3 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The lysate was centrifuged at 10,000 g at 4 °C for 15 min and the supernatant was used for purification of galectins by affinity chromatography.

Purification of Galectins by Affinity Chromatography—The supernatant of the cell lysate was passed over a lactosyl-Sepharose column (prepared as described by Leffler et al. (2)) equilibrated with the lysis buffer without protease inhibitors. Unbound material was washed off the column with buffer containing 135 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 5 mM β-mercaptoethanol. Lectin was eluted with buffer containing 150 mM lactose, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.5.

Immunoprecipitation—Galectin-3 was immunoprecipitated from both T84 cell lysate or from galectin-containing fractions eluted from lactosyl-Sepharose using anti-galectin-3 and protein G-Sepharose as described (20). All samples were preclarified twice with Sephardase CL-2B before the addition of the primary antibody. The washed precipitates were boiled in SDS-polyacrylamide gel electrophoresis sample buffer and separated on a 12% polyacrylamide-SDS gel. Proteins were localized by Coomassie Blue staining followed by fluorography at −70 °C with Kodak XAR-5 film and intensifying screens.

DNA Cloning, Manipulation, and Analysis—Unless specified otherwise, all manipulations of nucleic acids such as restriction, ligation, transformation, gel electrophoresis, blotting, gel elution, radiolabeling, and preparation of buffers were done using standard protocols (23). Samples were sequenced using Sequenase version 2.0 kit (U. S. Biochemical Corp.). The reported sequence was confirmed on both strands.

Human genomic DNA was amplified with convenant oligonucleotide primer pairs complementary to several different parts of the rat galectin-4 gene (3). A PCR using 1 μg of human genomic DNA (CLON-TECH/50-μl reaction. Reactions were carried out under conditions described by Gitt et al. (24) for 45 cycles: 40 s at 95 °C, 1 min at 55 °C, and 4 min at 72 °C. PCR using primers 5'-CGCCATGTCGAC-CCCGATCTTCAA-3' and 5'-ATGATGGTCTCTGGCCGTCG-3' yielded a major product that was cloned into the pCR1000 vector (Invitrogen, San Diego) according to the manufacturer’s instructions. This product was the same fragment containing an intact galectin-4 sequence. A human gene-specific antisense primer (HL36A, 5'-TGAGCCCTTGCCGGACCC-3') was designed based on the 5' exon sequences, to be used in PCR of reverse transcribed RNA.

Total RNA was isolated from confluent T84 cells using RnaZol (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. The RNA was reverse transcribed, using Moloney murine leukemia virus reverse transcriptase, with the C55 primer (5'-GACCTGAGTCATCGATT TTTTTTTTTTTTTTTTTT-3'), which is designed to hybridize with poly(A) and to provide an anchor sequence for use in subsequent PCR. The 20-μl solution was then diluted to 1 ml with 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 5 μl of this diluted solution was used in PCR with the antisense primer HL36A described above, and a sense primer (5', 5'-TTCTTATGAACTGCCGGACCC-3') designed based on further upstream sequence of rat galectin-4. The PCR conditions were: 5 cycles of annealing at 50 °C for 1 min and 40 cycles of annealing at 60 °C for 1 min, and extension at 72 °C was for 2 min in all cycles. One pure band was obtained, cloned into pCR1000, and sequenced using vector-specific primers. Based on this sequence, a sense human-specific primer, HL36B, 5'-TACCCCTGGTCCCGGACATTG-3', was synthesized, and PCR was performed with primers HL36B and C19 (a primer containing the anchor sequence (non-oligot(dT)) of primer C35) and cDNA as template. Annealing was at 56 °C for 1 min, and polymerization was at 72 °C for 1.5 min. A single band was obtained and ligated into pCR1000, yielding a clone that was used as a probe to screen a ZAP-II cDNA library prepared from T84 cells (a generous gift from Dr. J. R. Riordan, Salk Institute, La Jolla, CA). Positive clones were purified by rescreening. One positive clone was found to contain all previously identified sequence and additional 5' sequence. Subsequently a gale-

1 The abbreviations used are: PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLP, paraformaldehyde-peridate-lysine; BSA, bovine serum albumin.
Galectin-3 and Galectin-4 in T84 Cells

Fig. 2. Sequence of human galectin-4. Panel a, cDNA sequence with deduced amino acid sequence. Panel b, comparison of deduced amino acid sequences of human galectin-4 from T84 cells (hGal-4), rat galectin-4 (rGal-4; Ref. 3), and porcine galectin-4 (pGal-4; Ref. 30). Dots signify the same residue as in human galectin-4; dashes represent gaps introduced for alignment. The residues that are conserved in most galectins and known to interact with bound lactose (1) are indicated by asterisks.

assay plate was treated with solutions of laminin (5 μg/cm²), BSA (2%) or rat recombinant galectin-4 (5 μg/cm²) in PBS. After the addition of 50 μl to the wells, the plates were covered with Saran Wrap and incubated for 12 h at room temperature. Coated wells were washed for 10 min three times with PBS, and nonspecific adhesion sites were blocked by incubation with 2% BSA in PBS for 2 h at room temperature, followed by three 10-min washes with PBS.

Subconfluent T84 cell cultures were labeled with 10 μCi/ml [35S]methionine/cysteine as above, rinsed quickly with Ca²⁺- and Mg²⁺-free PBS, and gently dissociated to single cells and small cell clusters by incubation for 1 min at 37 °C with 0.05% trypsin solution containing 55mM EDTA.
0.02% EDTA, prewarmed to 37 °C. The cells were rinsed once in the growth medium and resuspended in the fresh growth medium to 4 × 10^6 cells/ml. The viability of cells as assessed by trypan blue exclusion was >99%. 300 μl of cell suspension was added to fill each well, and wells were sealed with an adhesive tape, avoiding formation of air bubbles. The plates were centrifuged at 24.9 × g for 8 min at 4 °C to bring the cells to contact with the substrate (“spin on”); after incubation for 30 min at 37 °C, the plates were inverted and centrifuged at 100 × g, for 8 min at 4 °C, to dislodge the nonadhering cells from the substrate (“spin off”). The plates were then quick-frozen in a dry ice-ethanol mix. The bottom 3 mm of each well was excised, and the adhering cells were quantified by scintillation counting in the presence of scintillation fluid. The total amount of applied cells (100%) was obtained by quantitation of cells remaining at the bottoms of BSA-coated wells immediately after the first centrifugation (spin on).

The conditions used were studied to use the inhibition of T84 cell adhesion by various saccharides, except that in this case a 2 mM concentration of the mixture was added to the wells with immobilized galectin-4, laminin, or BSA.

RESULTS

Identification of Galectin-4 and Galectin-3 in T84 Cells—To study the localization and function of galectins in a cell culture model of intestinal epithelium, we looked for these lactose-binding proteins in T84 colon carcinoma cells by affinity chromatography of cell extracts on lactosyl-Sepharose. We found that T84 cells express high concentrations of a 36-kDa and a 29-kDa galectin (Fig. 1, lanes A and B). The former reacted with anti-rat-galectin-4 on Western blots (Fig. 1, lane C), and the latter was immunoprecipitated by monoclonal anti-galecin-3 antibodies (Fig. 1, lane D).

The expression of galectin-4 in T84 cells was confirmed by cDNA cloning and sequencing (Fig. 2a). The amino acid sequence of the encoded T84 cell protein showed about 80% sequence identity with rat and porcine galectin-4 within the carbohydrate binding domains and about 50% sequence identity within the link region, and it contained all residues that are typically conserved in galectins and known to be associated with carbohydrate binding activity (Fig. 2b). From these data we conclude that the 36-kDa lactose-binding protein of T84 cells is human galectin-4.

Recently, sequences of a large number of randomly picked cDNA clones (expressed sequence tags) (25) from a T84 cell cDNA library have become publicly available. A search of these revealed that out of about 9,000 available sequences, 23 correspond to galectin-4, and 8 correspond to galectin-3, but no sequence corresponds to any other galectin. This provides further evidence that galectin-3 and galectin-4 are the main, or maybe sole, galectins expressed by T84 cells.

High Level of Expression of Galectins-4 and -3 in T84 Cells—The relative combined amount of galectin-3 and -4 in T84 cells was estimated as the amount of [35S]methionine/cysteine incorporated in the galectins compared with total trichloroacetic acid-precipitable protein from 2.5% Triton X-100 cell lysates. The combined radioactivity incorporated into the two galectins represents 1.4% of the trichloroacetic acid-precipitated radioactivity in early confluent cells but declined to about 0.5% in aging cells. Densitometry of SDS-polyacrylamide gel electrophoresis of the purified galectin mixture showed that galectin-4 accounts for 38–60% of the total galectin content.

The molar concentration of the galectins in T84 cells was calculated based on the absolute yield of lactosyl-Sepharose-purified galectins from a given area of confluent monolayer. Thus, about 0.5 mg of galectin was obtained from 20 culture plates (10-cm diameter), corresponding to a cell monolayer area of 800 cm². Since the cells are on average 25 μm high (Fig. 3), this corresponds to a cell volume of 2 ml. Hence the combined concentration of the galectins is 0.25 mg/ml or about 8 μM.

Based on the relative amounts of each galectin estimated by densitometry, the average concentration of galectin-3 and -4 in T84 cells is about 5 and 3 μM, respectively.

FIG. 3. Localization of galectin-3 and galectin-4 in a confluent monolayer of T84 cells by laser scanning confocal microscopy (XZ sections). The cells were fixed with PLP (panel a) or paraformaldehyde (panel b) for 12 h, permeabilized with 0.025% saponin at room temperature for 10 min, and analyzed after double immunolabeling. Apical galectin-3 is visible as green fluorescence (fluorescein-conjugated streptavidin) at the top of each panel, and the basal galectin-4 is marked by the presence of a red immunofluorescence (Texas Red-conjugated secondary antibody) at the bottom. Both bars, 25 μm.

Conditions for Immunocytochemistry of Galectins—The distribution of the two galectins in T84 cells was examined by immunocytochemistry and confocal microscopy. However, the immunocytochemical detection of the galectins was complicated by an unexpected difficulty in galecin fixation. Application of standard fixation and permeabilization conditions resulted in very weak staining of both galectins, suggesting that the galectins, which are very abundant in T84 cells, were lost because of poor fixation. Indeed, both galectins were found in the solutions used for cell permeabilization and could be purified on lactosyl-Sepharose, showing that they retain activity. It was particularly notable that active galecin could be identified that way even in methanol and acetone solutions (lyophilized and resolubilized in lysis buffer) collected after cell “fixation” for 5 min at −20 °C. Therefore, several permutations of different fixation and permeabilization procedures were tested which included prolonged fixation (12 h with 4% PLP or with 4% paraformaldehyde) followed by mild brief permeabilization with either saponin (0.025% in PBS, 10 min) to visualize intracellular galectins in confluent cells, or standard PLP-fixation without permeabilization to visualize membrane-associated galectins in EGF-treated confluent and in subconfluent cells.

Immunocytochemical Localization of Galectin-4 and -3 in Confluent T84 Cells—Fig. 3a presents the localization of galecin-4 (red) and galecin-3 (green) in 25-μm-tall confluent T84 cells as XZ sections after prolonged PLP fixation and brief saponin permeabilization. Galectin-4 is seen mainly as a 2-μm-thick layer near the basal membrane. Galectin-3 is mainly found in subapical accumulations revealed more clearly after fixation with paraformaldehyde instead of PLP (Fig. 3b). XY sections confirmed the apical-basal polarity of galecin distribution and also revealed significant amounts of diffusely distributed galectins (Fig. 4). Thus, panel a of Fig. 4 recorded at the level of the apical membrane (26 μm above the substratum) shows mainly galectin-3, whereas panel c recorded 1 μm above the substratum shows mainly galectin-4.
The XY sections also revealed particularly dense accumulations of the two galectins with a distinct morphology at the apical membrane (Fig. 4a, arrowheads), which were not seen readily in the XZ sections. Higher magnification of these detergent-resistant apical accumulations of galectins (Fig. 4d) demonstrates their very defined shape and composition. Galectin-4 is concentrated into circular accumulations with a large adjacent zone of galectin-3 either in the cell center (large arrowhead) or at the site of cell-cell contacts (small arrowhead) at the apical membrane (Fig. 4d). There is usually one organized accumulation of galectins in each confluent cell, and such formations have not been found in nonconfluent cells.

Immunocytochemical Localization of Galectin-4 in Confluent Calcium-depleted T84 Monolayers—The galectin-4 localization at the adherens junctions in porcine tongue squamous epithelium (5, 30) was not observed in the permeabilized confluent T84 monolayers. Since this might be due to the difficulties of fixing galectin-4 to its neighboring proteins resulting in its loss from permeabilized cells, as discussed above, we used an alternative approach to look for lateral localization of galectin-4 without permeabilization. To gain access to the extracellular adherens junction area in confluent T84 cells without permeabilization, the tight junction complexes were uncoupled by brief removal of Ca\(^{2+}\) with EGTA followed by fixation with PLP.

These EGTA-treated confluent monolayers were double immunolabeled for galectin-4 (red), and cytokeratin (green, Fig. 5). Cytokeratin immunostaining was used here as an indicator of the ability of antibodies to gain access to the interior of nonpermeabilized T84 cells; indeed, the anti-cytokeratin antibodies stained a layer about 2 \(\mu\)m inside the cell perimeter, outlining each cell, indicating that antibodies can penetrate nonpermeabilized T84 cells.

In the cells treated with EGTA for 15 min, galectin-4 is mainly observed as dramatic red patches (Fig. 5a, arrowhead) covering a large (8–16-\(\mu\)m long) lateral membrane area of cells next to a site of breakage in the monolayer. Following removal of Ca\(^{2+}\) with EGTA for 30 min (Fig. 5b), cell shapes were much less regular, and there were many more patches of galectin-4 covering the lateral membranes (Fig. 5b, arrowheads), again mostly next to a site of breakage in the monolayer. There was little if any colocalization of cytokeratin with galectin-4. Instead, in these conditions galectin-4 seemed to be localized more toward the cell periphery than cytokeratin, or extracellularly. These characteristic lateral galectin-4 patches were never seen in confluent monolayers that had not been treated with EGTA, but they persisted upon permeabilization.

Since in porcine oral squamous epithelium galectin-4 colocalizes with E-cadherin (5), we compared the localization of galectin-4 (red) and the intracellular domain of E-cadherin (green; Fig. 6, a–d) in EGTA-treated confluent T84 cells. After 30 min of EGTA treatment, the main galectin-4 staining was found near the apical membrane (XY plane 24 \(\mu\)m above glass level; Fig. 6a), concentrated in brightly staining crescent-shaped aggregations. These crescents are found at sites of the characteristic apical rounding and separation from neighboring cells and correspond to the lateral galectin-4 patches shown in Fig. 5, a and b. Less intense galectin-4 staining was found in the form of similar crescents 8 and 16 \(\mu\)m below the apical membrane, where most E-cadherin was found in the form of intracellular vesicular staining that did not colocalize with...
Galectin-3 and Galectin-4 in T84 Cells

Galectin Distribution in Subconfluent T84 Cells—The distribution of galectin-4 and galectin-3 in T84 cell cultures at different stages after seeding was examined by immunocytochemistry of fixed but not permeabilized cells. For these experiments, 2–3-day-old subconfluent cells were dissociated by brief and mild trypsin (0.05%) and low EDTA (0.02%) treatment for 1 min at 37 °C. These brief and gentle conditions were chosen since dissociation with a higher concentration of EDTA or EGTA results in a decreased rate of cell attachment and great loss of observable galectin-4. The cells were examined at 2, 12, 24, and 48 h after seeding.

An XZ section through a typical cell cluster 2 h after seeding is shown in Fig. 7a. In this cluster two cells are attaching to the substrate, and the top cell has not yet attached. A horizontal optical section through the top cell (Fig. 7b) shows a remarkable concentration of galectin-4 within one hemisphere of the cell periphery, whereas galectin-3 is distributed evenly around the cell periphery. This pattern was typical for rounded and not yet attaching cells. It is possible that this polarization of galectin-4 reflects the organization of the periphery of basal and apical cytoplasm (compare with Figs. 3 and 4) retained by the cells after dissociation from the polarized monolayer. In the two attached cells seen at the bottom of the cluster in Fig. 7a, galectin-4 is concentrated at the points of cell-substrate contact (arrowheads), whereas galectin-3 is found along a large part of the cell membrane. In 12-h- and 24-h-old colonies (Fig. 7, c and d), galectin-4 is again concentrated to small areas of the cell periphery at the same pole of pairs of newly divided cells (Fig. 7c) and at newly formed substrate contact sites at the colony periphery (Fig. 7d, small arrowhead). In contrast, galectin-3 is distributed more diffusely in the peripheral cytoplasm, including the vicinity of the membrane separating the newly divided cells. The galectin-4-positive patches that are often noticed at the surface of the glass (large arrowhead in Fig. 7d) are probably the remnants of attachment sites of cells dislodged during washes. The thin red line seen at the level of the glass in areas not covered by cells (Fig. 7a) may also be the result of adsorbed galectin-4 released from disrupted cells.

In 48-h cultures, large colonies have formed with cells rapidly proliferating and establishing first contacts with the substrate at the edge and more differentiated cells in the interior. Fig. 8 shows an overview of the galectin localization in such cultures revealed with immunoperoxidase instead of immunofluorescence to permit covisualization of the underlying cell and colony morphology. It is clear that both galectin-3 (panels...
Galectin-3 and Galectin-4 in T84 Cells

Galectin-3 and galectin-4 (panels b and d) are found in most lamellipodia at the edge of these fixed but not permeabilized cell colonies. In saponin- or methanol-permeabilized subconfluent cells, both galectins are essentially absent from lamellipodia (not shown), suggesting that their association with other cellular components at this location is detergent-sensitive in contrast to the galectin-4 seen at the lateral membrane of calcium-depleted cells shown in Fig. 5.

Double immunostaining and confocal microscopy of the 48-h cultures showed that both galectins are found within the same lamellipodia but in clearly distinct regions (Fig. 7, e and f); galectin-4 is always concentrated in the leading edge, whereas galectin-3 is found distributed more diffusely in the more proximal part. This distribution is preserved both in the compact, pointed lamellipodia typical for serum-starved cells (Fig. 7e) and in the wide flat (1–2-μm-tall, Fig. 7f, inset) but delicate and webby outgrowth areas seen 15 min after serum stimulation. Hence, the distribution of galectins defines three areas of lamellipodia: a leading edge occupied exclusively by galectin-4, the central area where the two galectins colocalize, and a proximal region rich in galectin-3.

In conclusion, in subconfluent T84 cells, galectin-4 is found...
in attachment sites of newly seeded cells and at the leading edge of lamellipodia. In contrast, galectin-3 is distributed along most of the cell periphery of these cells and is more concentrated in the posterior part of lamellipodia. Nuclear accumulation of galectin-3, observed previously in proliferating 3T3 cells (31) and normal colon epithelial cells (32), was not observed in T84 cells; in this regard T84 cells resemble other colon carcinoma cells (32).

Rat Recombinant Galectin-4 Enhances Adhesion of T84 Cells—Specific accumulation of galectin-4 in the cell-substrate contact sites in attaching cells, in lamellipodia of growing cells, and at the basal membrane of confluent monolayers suggests its involvement in cell-substrate adhesion. As the first attempt to investigate this possibility, we tested the ability of surface-adsorbed rat recombinant galectin-4 to support adhesion of T84 cells, using the procedure of McClay et al. (28).

The subconfluent T84 monolayers were dissociated to single cells and small cell clusters (such as shown in Fig. 7a) and brought into contact with microtiter wells coated with rat recombinant galectin-4, laminin, or BSA. After a 30-min incubation at 37 °C followed by centrifugation of the inverted microtiter plates at 100 g for 8 min at 4 °C, about 19% of all cells remained attached to galectin-4, compared with less than 1% of cells bound to BSA, and 65% cells attached to laminin (Fig. 9A). Hence, galectin-4 supports significant T84 cell adhesion, implying that it interacts with one or more receptors at the T84 cell surface.

The adhesion of cells to immobilized galectin-4 could be inhibited by lactose in a dose-dependent manner with 2.5 mM giving about half-maximal inhibition (Fig. 9B). Cellobiose was used as a control because it differs from lactose only by the stereochemistry of the 4'-OH (axial in lactose, equatorial in cellobiose) but does not bind galectins (1, 33). Cellobiose was at least 100-fold less potent as an inhibitor of T84 cell binding to galectin-4. The binding of T84 cells to laminin was inhibited 10% by 25 mM lactose and not at all by 25 mM cellobiose. This shows that the cells were sufficiently intact to adhere and not affected deleteriously by nonspecific effects of these saccharide concentrations.

**DISCUSSION**

This paper describes the different locations of two very abundant galectins, galectin-3 and -4, in T84 cells in the subconfluent state and after differentiation into a polarized epithelium. In confluent polarized cells the two galectins accumulate at opposite poles, with galectin-3 at the apical and galectin-4 at the basal membrane. In subconfluent cells they concentrate in different parts of lamellipodia, with an accumulation of galectin-4 at the leading edge and galectin-3 localized more proximally.

Concentration of these galectins in defined subcellular areas has been observed earlier both in cultured cells and in tissues. Apical localization of galectin-3 in T84 cells agrees well with the similar finding in other polarizing epithelial cell lines (10,
11) and in kidney epithelium (34). Galectin-4 has been found previously in globular structures at the cell periphery corresponding to areas of adherens junctions in oral epithelium (5) and in aggregates at the apical membrane of lumenal cells in esophageal epithelium (6).

The natural ligands for galectins are thought to be β-galactosides, and the differences in their binding specificity for complex saccharides (2, 3, 33) would be sufficient to explain differential targeting. However, since β-galactosides are not detectable in the cytoplasm, the intracellular galectin ligands must be of another nature, probably proteins. Indeed, interaction between galectin-3 and a nuclear glucose-binding protein of 70 kDa (CBP70) has been demonstrated (35), and most recently galectin-3 was shown to associate with Bcl-2, a well known intracellular suppressor of apoptosis (36). Both of these associations occur via protein-protein interactions but are inhibited by lactose, indicating direct or indirect involvement of the galectin carbohydrate binding site. The association of galectin-3 with cytoplasmic and nuclear ribonucleoprotein complexes might be a lipid. First, detergent treatment of PLP-fixed cell monolayers might enable it to cross-link glycoconjugates at the cell surface and/or in the extracellular matrix. 2) Galectin-4 is externalized at the basolateral surface of polarized cells including T84 (38). 3) Surface-adsorbed recombinant galectin-4 induces adhesion of T84 cells (Fig. 9) in a lactose-sensitive way.

The limited effect of lactose on binding of T84 cells to laminin (a common measure of cell-substratum adhesion) does not rule out a role for galectin-4 in cell adhesion. Its role may be in an earlier stage or a different aspect of the complex cell adhesion process than is measured by this assay. For example, the sharply demarcated galectin-4 accumulations observed at the sites of breaks in the monolayer after Ca²⁺ depletion and at the tips of lamellipodia invites the speculation that it helps in initial reattachment and/or spreading of cells in a disrupted cell monolayer. Morphologically similar lamellipodia-like extensions and localized lateral membrane changes form quickly around the edges of a wound, either experimentally induced in a T84 monolayer (39) or in vivo during restitution of the intestinal epithelium (40, 41). In this way, galectin-4 could play an important role in the maintenance of epithelial integrity and in the epithelial wound healing process.

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