Induction of Terminal Differentiation in Epithelial Cells Requires Polymerization of Hensin by Galectin 3✪

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Abstract. During terminal differentiation, epithelia become columnar and develop specialized apical membrane structures (microvilli) and functions (regulated endocytosis and exocytosis). Using a clonal intercalated epithelial cell line, we found that high seeding density induced these characteristics, whereas low density seeding maintained a protoepithelial state. When cells were plated at low density, but on the extracellular matrix of high density cells, they converted to the more differentiated phenotype. The extracellular matrix (ECM) protein responsible for this activity was purified and found to be a large 230-kD protein, which we termed hensin. High density seeding caused hensin to be polymerized and deposited in the extracellular matrix, and only this form of hensin was able to induce terminal differentiation. Antibodies to hensin blocked the change in phenotype. However, its purification to homogeneity resulted in loss of activity, suggesting that an additional protein might be necessary for induction of terminal differentiation. Here, we found that a 29-kD protein specifically associates with hensin in the ECM. Addition of purified p29 restored the activity of homogenously purified hensin. Mass fingerprinting identified p29 as galectin 3. Purified recombinant galectin 3 was able to bind to hensin and to polymerize it in vitro. Seeding cells at high density induced secretion of galectin 3 into the ECM where it bundled hensin. Hence, the high density state causes a secretion of a protein that acts on another ECM protein to allow the new complex to signal the cell to change its phenotype. This is a new mechanism of inside-out signaling.

Key words: terminal differentiation • inside-out signaling • hensin • DMBT1 • galectin

Introduction

The plasma membranes of epithelial cells, the most ancient differentiated cells of metazoa, are polarized into apical and basolateral domains, each of which have different protein and lipid compositions. Their cytoplasm is also polarized, with the nuclei occupying the basal half of the cell, whereas the Golgi apparatus is located in a subapical region. The cells are attached to each other by a variety of junctions, and they synthesize their own extracellular matrix (Yeaman et al., 1999). Despite the fact that all epithelial cells share these characteristics, even a superficial examination of different organs demonstrates the presence of many easily recognizable types of epithelial cells. This implies that development and differentiation of epithelia proceeds in at least two distinct steps: the conversion of a nonepithelial cell to a protoepithelium, followed by a process of terminal differentiation. Protoepithelial cells exhibit the fundamental properties of epithelia, but perhaps are still able to divide and migrate. Terminally differentiated epithelia are stationary and do not proliferate, but acquire many type-specific features, such as brush borders, specialized organelles (such as storage granules) with the ability for regulated exocytosis, characteristic cell shape (e.g., columnarization), and other tissue-specific properties. Terminal differentiation continues to occur in adult animals in the intestine, skin, prostate, and other organs. Recent studies in many organisms have shown that interruption of such differentiation programs leads to unbridled growth leading to or contributing to the development of cancer.

Our work on terminal differentiation started from a seemingly unrelated finding that dealt with the mechanism of polarized distribution of the vacuolar H^+-ATPase and the band 3 anion exchange protein, kAE1, in the intercalated cell of the kidney. We found that these cells exist in two forms, the β form, kAE1, was present in the apical membrane, whereas the ATPase was located in the basolateral membrane. In the α form, these proteins were located

✪The online version of this article contains supplemental material.

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on the opposite cell membranes. Remarkably, we found that both in vitro (van Adelsberg et al., 1994) and in vivo (Schwartz et al., 1985) the β cell could convert to an α phenotype. When a clonal intercalated cell line was seeded on filters at subconfluent density and examined at confluence, the cells exhibited all the characteristics of the β phenotype. However, when the same clone of cells was seeded at confluent density and examined a day later, the cells were of the α phenotype. High density cells were solubilized and discarded and new cells were seeded at low density on these "conditioned" filters. These cells were instructed to change to an α type. We purified a protein from the extracellular matrix of high density cells that was capable of inducing the conversion of polarity, and we termed this protein hensin (van Adelsberg et al., 1994; Takito et al., 1996). Remarkably, the hensin-induced changes were not restricted to the reversal of the polarized distribution of kAE1 and the H^+-ATPase. Rather, there was a large morphological transformation of the low density phenotype from a flat shape to a columnar cell. Furthermore, low density cells had no apical microvilli and they did not express the cytoskeletal proteins that cause the appearance of microvilli, such as villin, cytokeratin 19, or a subapical actin cytoskeleton. Hensin induced the appearance of exuberant microvilli, localized actin to the subapical region, and the appearance of apical villin and cytokeratin 19 (Vijayakumar et al., 1999). Finally, low density cells had no apical endocytosis or exocytosis, whereas high density cells had vigorous apical endocytosis and had been shown previously to regulate apical exocytosis. Antibodies to hensin blocked all of these changes in phenotype. These findings demonstrate that the hensin-mediated conversion of the low density to high density phenotype is actually terminal differentiation.

Cloning of the hensin cDNA showed that it was composed of eight scavenger receptor cysteine-rich (SRCR)[1] domains (Resnick et al., 1994), two complement subcomponents Clr/Cls, Uegf, Bmp1 (CUB) domains (Bork and Beckmann, 1993), and one Zp domain (Bork and Sander, 1992). Five other proteins have now been sequenced all of which were composed of these three domains, but in different combinations (Li and Snyder, 1995; Cheng et al., 1996; Mollenhauer et al., 1997; Holmskov et al., 1999; Matsushita et al., 2000). We recently discovered that all of these transcripts were derived from a single gene by alternative splicing (Takito et al., 1999). Hensin is expressed in all epithelia tested, whereas DMBT1 is expressed in the brain and gp340 in lung macrophages. The gene is deleted in a large fraction of epithelial and neural tumors (Mollenhauer et al., 1997; Mori et al., 1999; Takeshita et al., 1999; Wu et al., 1999). Given that interruption of terminal differentiation pathways is often invoked as a cause of malignant transformation, these findings raise the possibility that hensin, or its alternately spliced products, act as tissue-specific tumor suppressors.

We showed previously that hensin exists in many forms. It is secreted as a soluble monomer, whereas in the extracellular matrix (ECM) it exists as a high order multimer. Only multimeric hensin of the ECM could induce a change in its target cells (Hikita et al., 1999). Addition of exogenous monomeric hensin to high density cells caused a change in its polymerization state, converting it to its dimeric and tetrameric forms. These studies demonstrate that "activated" (i.e., high density) cells were capable of sending a signal to an extracellular protein, a phenomenon similar to inside-out signaling by integrin receptors (Hughes and Pfaff, 1998).

We found that only ECM hensin was capable of changing the phenotype of the cells, but when ECM hensin was purified to homogeneity, it lost activity (Takito et al., 1996). Here, we show that the polymerization of hensin into functional complexes (likely to be fibers) is mediated by a 29-kD protein that, when purified, was capable of restoring the ability of purified hensin to induce apical endocytosis of low density cells. Mass fingerprinting of the purified protein identified it as galectin 3, a member of a large family of lectins that recognize β-galactoside structures and expressed by many cell types (Perillo et al., 1998; Hughes, 1999). Interestingly, the only cell that expresses it in the adult kidney was the α-type intercalated cells, the high density equivalent (Winyard et al., 1997). High density cells secreted galectin 3 to the basal surface where it bound to and bundled hensin into a multimer.

Materials and Methods

Cells

Clone C of intercalated cells were maintained at 32°C, as described previously (Van Adelsberg et al., 1994). The cells were seeded on a 0.45-μm polycarbonate filter (Transwell; Corning Costar Corp.) at a density of $2 \times 10^4/cm^2$ (low density) or 4×10^5 cells/cm^2 (high density) and cultured at 40°C to inactivate the T antigen.

Preparation of Cell, Conditioned Media, and ECM Extracts

Cells grown at high or low density on filters were extracted with buffer A (1% Triton X-100 and 1 mM calcium chloride) for 1 h at 4°C on a rotary shaker. Cell extracts were removed and filters were scraped in this solution with a cell scraper to remove loosely attached materials. The filters were then washed thoroughly with the same solution for another hour at 4°C. Insoluble material remaining on these filters was extracted with 4 M guanidine hydrochloride, 50 mM sodium acetate, pH 6.5, 5 mM EDTA, and 0.5% CHAPS at 4°C overnight; this was referred to as the ECM fraction (van Adelsberg et al., 1994). Both cell and ECM extracts were dialyzed against 50 mM Tris-HCl, pH 8.0, at 4°C overnight. Conditioned media were collected and spun at 5,000 *g* for 5 min at 4°C to remove any remaining cells. The top two-third of supernatants was saved.

Immunoprecipitation

To cell extracts, ECM, or conditioned media, one-tenth volume of buffer B (1% SDS, 1% Triton X-100, 100 mM Tris-HCl, pH 8.0, 10 mM calcium chloride) and guinea pig anti-hensin serum was added at a dilution of 1:500, and immunoprecipitation was performed at 4°C for 1 h (Takito et al., 1996). Immunoprecipitates were collected by mixing with protein A–Sepharose CL-4B (Amersham Pharmacia Biotech) at 4°C for 1 h. Beads were washed three times with a buffer containing 0.1% SDS, 0.1 mM EDTA, 0.1% Triton X-100, and 10 mM Tris-HCl, pH 8.0. Immunoprecipitates were recovered from the beads by boiling in sample buffer for 3 min, and then they were subjected to SDS-PAGE. Gels were fixed with 10% acetic acid/10% methanol, soaked in Amplify solution (Amersham Pharmacia Biotech), dried, and exposed to x-ray film (Kodak X-OMAT; Eastman Kodak) at −80°C. Densitometric scanning was performed by a Computing Densitometer (model 300A; Molecular Dynamics).

Apical Endocytosis

Monolayers plated at various densities were exposed to HCO_3^-/CO_2–free medium containing 2–5 mg/ml horseradish peroxidase (Sigma-Aldrich)

[1]*Abbreviations used in this paper:* DMMA, dimethyl maleic anhydride; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); ECM, extracellular matrix; MALDI-reTOF MS, matrix assisted laser desorption ionization-reflectron time of flight mass spectrometry; SRCR, scavenger receptor cysteine-rich.

and 10% FBS on the apical side of the monolayer only. The cells were incubated for 10 min at 40°C or 4°C. At the end of the incubation, the cells were immediately transferred to ice-cold medium and were washed 10 times at 4°C on a rotary shaker. The monolayers were then solubilized in 1% Triton X-100, and the activity of horseradish peroxidase was measured by measuring the initial rate of hydrolysis of *O*-dianisidine (Sigma-Aldrich) in a Beckman DU-2 spectrophotometer. The volume of endocytosis was calculated by constructing a standard curve of horseradish peroxidase activity in dilutions of the starting material. Apical endocytosis was taken to be the difference between uptake at 40°C subtracted by uptake at 4°C. For viewing endocytosis under the fluorescence microscope, the cells were treated as described above, except that 10 mg/ml FITC-dextran (Sigma-Aldrich) was added instead of horseradish peroxidase. After the wash, cells were rinsed thoroughly with PBS containing 1 mM calcium chloride and 1 mM magnesium chloride. The filters were mounted on glass slides and viewed by a fluorescence microscope.

Chemical Cross-Linking

Cells grown on transwells at high density were labeled with 100 μCi/ml of [^{35}S]methionine/cysteine (^{35}S-protein labeling mix; DuPont NEN Express) for 12 h. The cells were extracted twice with buffer A at 4°C for 1 h and then washed with PBS. Chemical cross-linking was performed by soaking these filters in buffers containing 1 mM DTSSP (3,3′-dithiobis [sulfosuccinimidyl-propionate]; Pierce Chemical Co.). Cross-linking was terminated by 50 mM Tris-HCl, pH 7.5. ECM deposits were extracted from these filters, dialyzed against 50 mM Tris-HCl, pH 7.5, and then immunoprecipitated with anti-hensin serum, as described above. Samples were separated on 7.5% SDS-PAGE and then followed by fluorography. For analysis by two-dimensional gel electrophoresis, samples were first run on 7.5% SDS-PAGE in nonreducing condition. The gel strip was cut out and soaked in 100 mM Tris-HCl, pH 6.8, 1% SDS, 50 mM DTT, and 1% 2-mercaptoethanol for 30 min. This strip was laid on the top of 15% SDS–polyacrylamide gel. 1% agarose, 100 mM Tris-HCl, pH 6.8, 1% 2-mercaptoethanol, and 0.1%SDS was poured to fill the space between the strip and the running gel. 1 mM DTT was added to the running buffer only for the second dimension.

Preparation of Dimethyl Maleic Anhydride–treated ECM-coated Filters

In brief, high density cells seeded on transwells were extracted with buffer A, and filters were preequilibrated with a solution containing 50 mM Hepes-KOH, pH 8.5. 5 mM dimethyl maleic anhydride (DMMA; Sigma-Aldrich), dissolved in the same buffer, was added to the filters and incubated at 4°C for 1 h on a rotary shaker. The filters were washed thoroughly with 50 mM Hepes-KOH, pH 8.5, to remove excess DMMA, then and incubated with 50 mM Hepes-KOH, pH 6.7, at 4°C overnight. The filters were washed with PBS and used for cross-linking experiments. For apical endocytosis assays, DMMA-treated filters were incubated with 50 mM Hepes-KOH, pH 6.7, in the presence or absence of 100 g/ml his-tagged galectins at 4°C overnight, and they were then equilibrated with DME at 4°C overnight before cells were seeded on them at low density.

Density Gradient Analysis of Hensin

Conditioned media were collected from low density or high density cells and concentrated 10-fold using Centriprep-10 (Amicon), and they were divided into Eppendorf tubes and some were treated with galectin 3. The samples were loaded on 12 ml of 5–30% sucrose or 5–40% metrizamide gradients in 50 mM Hepes-KOH (pH 7.5, 8.5, or 6.7) and ultracentrifuged at 100,000 *g* for 16 h at 4°C. Proteins in each fraction (1 ml) were precipitated by 6% TCA, dissolved in a sample buffer, subjected to 7.5% SDS-PAGE, and then followed by Western blotting with anti-hensin serum.

Purification of p29

ECM was extracted using EDTA and 4 M guanidine HCl from 2×10^9 cells seeded on transwell filters at high density, as described previously (van Adelsberg et al., 1994; Takito et al., 1996). Insoluble materials were removed by centrifugation at 10,000 *g* for 15 min at 4°C. The supernatant was dialyzed extensively against 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, at 4°C. Aggregates formed during dialysis were removed by filtration (MILLEX-GV; Millipore). The sample was concentrated to ~30-fold using Centriprep-10 (Amicon). This material was loaded onto a Resource Q column (Amersham Pharmacia Biotech) preequilibrated with a solution containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 μM phenylmethylsulfonylfluoride, 0.3 μM aprotinin, 0.8 μM pepstatin A, and 1 μM leupeptin. The flow-through fraction was saved and dialyzed at 4°C against 50 mM MES, pH 6.5, and 1 mM EDTA with the same protease inhibitors mentioned above. The sample was loaded on a HiTrap Heparin column (Amersham Pharmacia Biotech) with the same buffer, and the bound proteins were eluted by a gradient of 0–500 mM NaCl in the same buffer, followed by a wash with 1 M NaCl. Eluates between 180 mM and 200 mM NaCl were collected, dialyzed against 50 mM Hepes-KOH, pH 6.7, and stored at 4°C until used.

Identification of p29

Protein bands were excised from the gels, digested with trypsin (Hellman et al., 1995), and processed for mass spectrometric fingerprinting, as described previously (Erdjument-Bromage et al., 1998). In brief, peptide mixtures were partially fractionated on Poros 50 R2 RP microtips, and the resulting peptide pools were analyzed by matrix assisted laser desorption ionization-reflectron time of flight mass spectrometry (MALDI-reTOF MS) using a Reflex III instrument (Brüker Franzen). Selected mass values were then taken to search a protein nonredundant database (NR; National Center for Biotechnology Information) using the PeptideSearch (Mann et al., 1993) algorithm.

Immunocytochemistry

Clone C cells were plated at high or low density and cultured at the indicated times at 40°C on transwell filters. In the studies to determine the extracellular accessibility of galectin 3, the filters were first incubated with 1:100 dilution of the rabbit anti-galectin 3 antiserum (provided by Dr. F.T. Liu, La Jolla Insitute for Allergy and Immunology, La Jolla, CA) and guinea pig anti-hensin serum in PBS for 4 h at 4°C, followed by the appropriate secondary antibodies for 1 h at 4°C. They were then washed with PBS, fixed in 4% paraformaldehyde, and incubated with 10–20 μg/ml of Hoechst 33342 dye or Sytox Green (Molecular Probes). The filters were washed extensively with PBS, mounted on glass slides with 90% glycerol in PBS and 0.1% phenylene diamine, and viewed by an axiovert 100 laser scanning confocal microscope (model LSM 410; Carl Zeiss, Inc.). Excitation was accomplished with an argon-krypton laser producing lines at 488, 568, or 647 nm. Rhodamine-labeled samples were viewed with the 568-nm channel (red), whereas the fluorescein/Hoechst 33322/Sytox green–labeled samples were viewed with 488-nm (green) channel. The images were collected at 1-μm-thick optical sections and analyzed by the Zeiss LSM-PC software. The final images were processed with Adobe Photoshop® software.

Sprague-Dawley rat colon was embedded and frozen in Tissue-Tek OCT Compound (Miles Laboratories) until cut into 5-μm cryostat sections. The sections were fixed in ice-cold methanol for 8 min, washed extensively with PBS, and blocked with 10% fetal calf serum and 1% donkey serum (Jackson ImmunoResearch Laboratories) in PBS for 30 min. The sections were then incubated with guinea pig anti-hensin antibody (Takito et al., 1996), diluted 1:100 in PBS, at room temperature for 2 h, and then incubated with rhodamine-conjugated donkey anti–guinea pig IgG. The colon sections were further incubated with rabbit anti-galectin 3 antiserum, diluted 1: 100 in PBS, and then by fluorescein labeled anti–rabbit IgG. Finally, the sections were extensively washed and mounted for analysis by confocal microscopy.

Cloning of Galectin 3 and Production of His-tagged Galectin 3

Rabbit galectin 3 was amplified by PCR from total RNA fraction of clone C cells with 5′ and 3′ primers (5′-CGCGGATCCATGGCGGATG-GTTTTTCGC-3′ and 5′-GGGTTCGAAAATATAGTATCGTACACT-TCG-3′, respectively). The PCR product was ligated into pCR2.1TOPO vector (Invitrogen). The fragment encoding galectin 3 was excised with BamHI and HindIII and was ligated to pQE30 (QIAGEN) at the same sites yielding pQE-Gal, which encodes His6-tag at the NH_2 terminus of galectin 3. Expression of his-tagged galectin 3 in *Escherichia coli* (strain JM109) was induced by 2 mM IPTG at 37°C for 5 h. His-tagged galectin 3 was purified from the bacterial lysate using Ni beads (QIAGEN). After elution from the resin with 100 mM imidazole, the eluate was dialyzed against PBS at 4°C and stored at −80°C. On immunoblots, the antibody recognized a single band of the same molecular mass as authentic his-tagged galectin 3 in both bacterial lysates and total cell extracts (see Fig. 3, B and C).

Online Supplemental Materials

An additional supplemental figure (Fig. S1) is available at http://www.jcb.org/cgi/content/full/151/6/1235/DC1

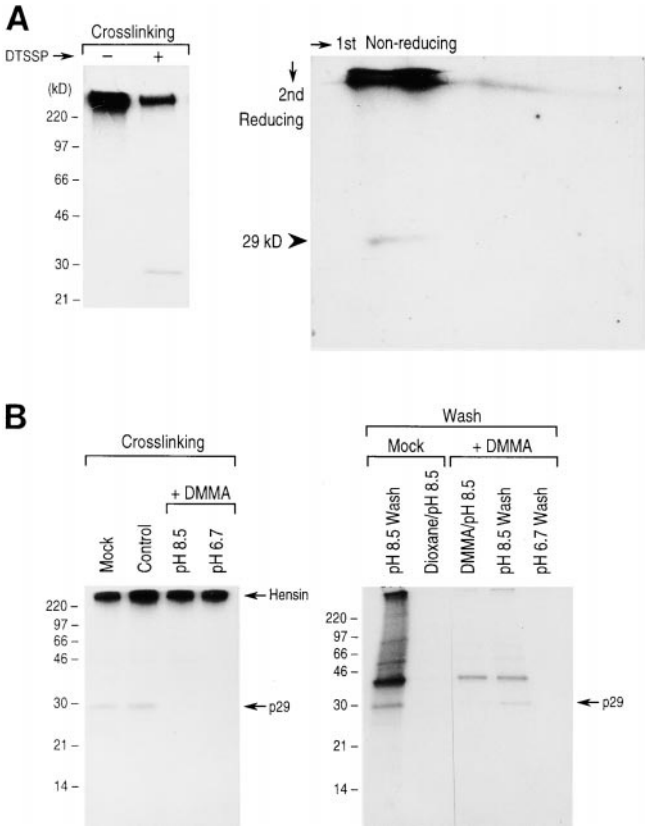

Figure 1. Cross-linking with DTSSP. (A) Cells were seeded at high density for 2 d and then labeled with [^{35}S]methionine/cysteine overnight. After removal of free amino acids, they were treated with basolateral DTSSP at 4°C for 1 h. The left gel shows that p29 can be cross-linked to hensin in the ECM. Immunoprecipitation with anti-hensin antibodies of the ECM is displayed on reducing SDS-PAGE. The right gel shows that p29 is released from hensin, by two-dimensional electrophoresis of a similar study to that on the left; the first dimension was nonreducing and the second was reducing. (B) The left gel shows that DMMA treatment prevents the association of p29 with hensin. High density cells were cultured for 2 d and labeled with [^{35}S]methionine overnight. Their ECM was cross-linked with DTSSP after the following treatments were performed: none (Mock), washed with pH 8.5 buffer (Control), washed with pH 8.5 buffer containing 1 mM DMMA (DMMA, 8.5), or washed with pH 6.7 buffer after being treated with DMMA at pH 8.5 (DMMA, 6.7). The ECM was then extracted and subjected to immunoprecipitation with anti-hensin antibodies, as described. The right gel shows that p29 is released from the ECM by DMMA treatment. The wash of the experiment shown in the left gel was precipitated by TCA and separated on SDS-PAGE. In Mock lanes, the ECM was washed sequentially with pH 8.5 buffer and the same buffer containing dioxane, the carrier for DMMA. Note that there is release of p29 in the first but not the second wash. After adding DMMA, removal of the solution that contained DMMA showed that there was some p29, but the next wash contained all of the p29. Further washes with pH 6.7 did not release more p29.

Results

Hensin and p29 Are Needed To Induce Terminal Differentiation

To purify hensin to homogeneity required harsh denaturing conditions (0.1% SDS and 4 M urea), and this fraction did not induce apical endocytosis (Takito et al., 1996). These results suggested that another protein might be required for its function, and we used cross-linking to identify potential binding partners. High density cells were labeled by [^{35}S]methionine/cysteine and exposed at 4°C for 1 h to the impermeant cleavable *N*-hydroxysuccinimide–based cross-linker DTSSP. The cells were then solubilized and discarded and their ECM was extracted by guanidine hydrochloride and subjected to immunoprecipitation using anti-hensin antibodies. A 29-kD protein (p29) was consistently found, with occasional additional appearance of two bands ~46 kD (Fig. 1 A, left). p29 was cross-linked to hensin only in the ECM fraction and not in cell lysates or in apical or basolateral media (not shown). To ensure that p29 is a genuine cross-linked product of hensin, two-dimensional gel electrophoresis was carried out, where the first dimension was nonreducing and the second was reduced by DTT and β-mercaptoethanol. It is clear from Fig. 1 A (right) that p29, which should be released from hensin only in the second dimension, originated from the high molecular weight hensin.

Few, if any, of the abundant proteins of the ECM were seen in immunoprecipitates of cross-linked hensin. To determine whether the hensin–p29 interaction was specific, we used two classes of reagents (sulfhydryl and DMMA), which we had previously shown could disaggregate hensin and prevent its ability to induce terminal differentiation (Hikita et al., 1999). DMMA is a reagent that reacts with the free amino group of lysine to produce a maleyl lysine, thereby converting it from a cation to an anion. This large change in electrostatic relationship frequently results in disaggregation of subunits (Means and Feeney, 1971). Maleyl lysine is stable at neutral or alkaline pH, but is rapidly hydrolyzed in acidic media, resulting in the regeneration of the cationic lysine. DMMA treatment reversibly converted hensin multimers to monomers (Hikita et al., 1999). If a complex of hensin and p29 indeed exists, then treatment with DMMA might be expected to dissociate the two proteins, allowing p29 to be washed away. Extensive washing of the ECM with pH 8.5 buffers initially led to the appearance of several proteins, including a 29-kD protein (Fig. 1 B, right gel, pH 8.5 Wash). Further washes with pH 8.5 buffer containing the carrier for DMMA, dioxane, did not lead to elution of more of the 29-kD protein (Fig. 1 B, right gel, Dioxane/pH8.5). Despite the fact that these excessive washes released a large amount of p29, cross-linking and immunoprecipitation revealed that there was significant p29 still bound to hensin (Fig. 1 B, left gel, Mock and Control). Washing at pH 8.5, after the addition of DMMA, led to the release of more p29 (Fig. 1 B, right gel, pH 8.5 Wash). Cross-linking of this ECM fraction, which was followed by immunoprecipitation with anti-hensin antibodies, showed that hensin was not cross-linked to p29 (Fig. 1 B, left gel, pH 8.5 or 6.7). Washing with pH 6.7 releases the DMMA from proteins and results in the refolding of hensin; however, this "refolded" hensin was no longer able to induce apical endocytosis (Hikita et al., 1999). Cross-linking of this refolded ECM hensin shows that it is no longer associated with p29 (Fig. 1 B, left gel, pH 6.7). Treatment of the ECM with DTT followed by *N*-ethyl maleimide to alkylate the free sulfhydryls before addition of the cross-linker also prevented the association of hensin and p29 (data not shown). These studies suggested

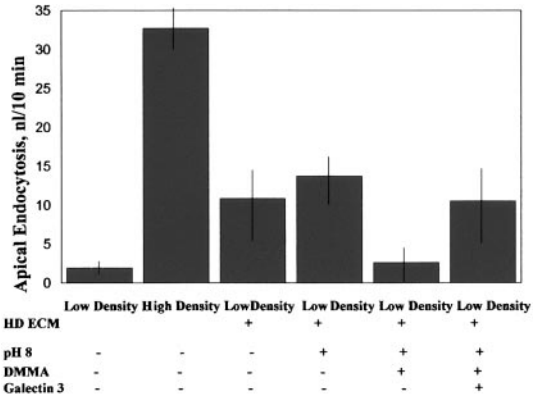

Figure 2. Apical endocytosis using HRP. Low density cells have no apical endocytosis, whereas high density cells have high levels. The high density ECM induces apical endocytosis in cells seeded at low density. Washing the high density ECM with the carrier for DMMA (pH 8 and dioxane) does not disrupt the effect of the high density ECM on low density cells. However, treatment of the high density ECM with DMMA inhibits its ability to induce apical endocytosis in low density cells. Addition of galectin 3 purified from high density ECM to the DMMA-treated high density ECM results in recovery of its ability to induce apical endocytosis in low density cells. Results are given as the mean ± SEM.

that hensin binds to, or is at least the nearest neighbor, of p29, but only when hensin is located in the ECM and in a native conformation.

Apical endocytosis was measured (Fig. 2) and we found, as before, that low density cells showed no apical endocytosis, whereas high density cells had vigorous apical endocytosis. Seeding cells at low density, but on high density ECM, induced apical endocytosis. Treatment of the ECM with DMMA followed by extensive washing also inhibited its ability to induce apical endocytosis, whereas washes with pH 8, but without DMMA treatment, had no effect (Fig. 2). Even when DMMA was removed (by treatment with pH 6.7), the refolded hensin was incapable of inducing apical endocytosis. These studies suggest that the interaction of hensin with p29 is necessary for its activity.

Interestingly, the hensin–p29 association was greatly diminished when cross-linking was performed a week after the cells were seeded. Although the amount of hensin synthesized on day 7 after seeding was 66% of that on day 2, the amount of p29 cross-linked was decreased to 28% on day 7 compared with day 2 (Fig. 3 A, left gel). In pulse-chase studies, cells were labeled 2 d after seeding and the ECM cross-linked 3 or 7 d later. We found that hensin was associated with p29 during the early period decreasing 1 wk later (Fig. 3 A, right gel). Taken together, these results suggest that p29 interacts with hensin only in the early stages after high density seeding. However, this conclusion assumes that the access of the cross-linking reagent to the hensin–p29 complex, at later times, is quantitatively similar to its access at earlier times. Regardless, the critical information is that hensin and p29 associate early. Hensin is localized to the ECM very early after seeding, and we showed previously that induction of apical endocytosis occurred within 24 h of seeding the cells at high density (Van Adelsberg et al., 1994). These results demonstrate that the nearest neighbor of hensin in the ECM is a 29-kD protein whose association with hensin is inhibited by procedures that unfold it and reversibly disaggregate hensin. Furthermore, it appeared that this association occurs at a time that is critical for the change in phenotype induced by high seeding density.

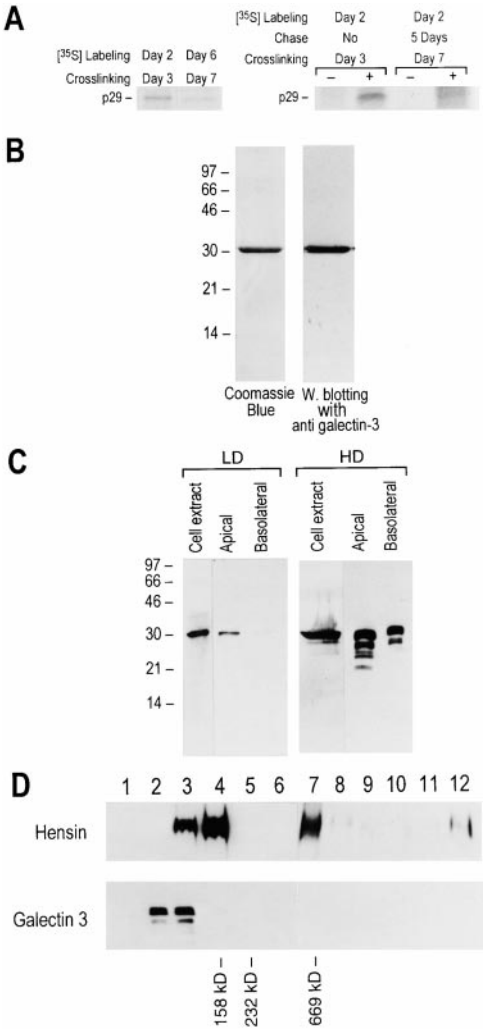

Figure 3. Characteristics of the binding of p29 (galectin 3) to hensin. (A) The left gel shows cells were labeled with [^{35}S]methionine on day 2 or 6 after seeding at high density. They were then cross-linked with the impermeant reagent DTSSP on day 3 or 7 after seeding. ECM proteins were then immunoprecipitated with anti-hensin antibodies. The right gel shows cells were labeled on day 2 after seeding and chased for 0 or 7 d. The cross-linking and immunoprecipitation was performed on day 3 or 7 after labeling. (B) Bacterially expressed his-tagged galectin 3 was purified on affinity columns and stained with Coomassie blue (left gel). The material reacted with anti-galectin 3 polyclonal antibody (right gel). (C) Cell fractionation of low density (LD) and high density (HD) cells are shown, followed by immunoblotting of total proteins using anti-galectin 3 polyclonal antibodies. Cells were extracted with Triton X-100, and the apical and basolateral media were precipitated and separated on SDS-PAGE. (D) Sucrose density gradient centrifugation of media from high density cells probed with anti-galectin 3 and anti-hensin antibodies.

p29 Is Galectin 3

We purified p29 from high density ECM by first solubilizing the cells with Triton X-100 after 2 d of seeding at high density. The detergent was discarded. The ECM fraction

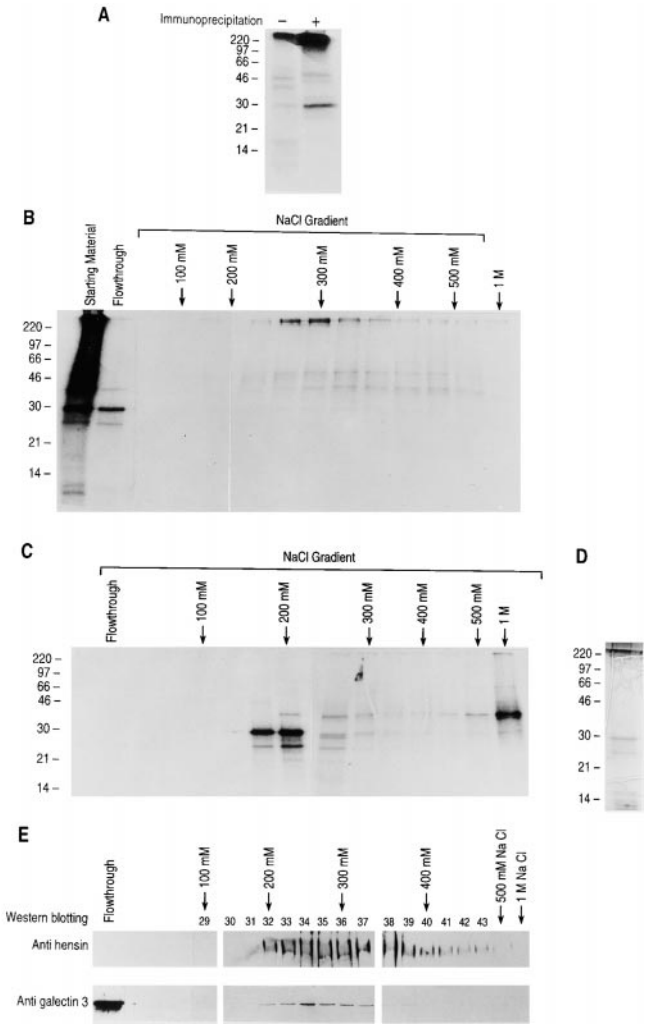

Figure 4. Purification of p29. (A) The ECM contains a 29-kD protein that has the same mobility as the cross-linked p29. ^{35}S-labeled fractions were used in this study. (B) p29 does not bind to anion exchange Q resin. Note that all 29-kD proteins were in the flow through, and hensin is eluted in the 200–400 mM NaCl fractions. ^{35}S-labeled fractions were used in this study. (C) Heparin-Sepharose chromatography of the Q-resin flow through. Most of the p29 eluted in the 100–200 mM NaCl fractions. ^{35}S-labeled fractions were used in this study. (E) Silver-stained gels of the combined two fractions containing p29. (E) Although p29 does not bind to the Q resin, it is present in the hensin-containing fractions of the eluate. Immunoblots of the fractions are shown in B above.

that remained on the polycarbonate filter was then extracted with EDTA and 4 M guanidine hydrochloride. This material contained a 29-kD protein that had a mobility identical to the protein that was cross-linked to hensin (Fig. 4 A). After dialysis to remove guanidine, the ECM proteins were subjected to anion exchange and then heparin sepharose chromatography (Fig. 4). p29 did not bind to the Q resin, but it did bind to heparin from which it was eluted with 200 mM salt (Fig. 4, B and C). We concluded that the 29-kD protein isolated from the high density ECM was the same as that cross-linked to hensin for the following reasons. First, the two proteins have an identical mobility on SDS-PAGE (Fig. 4 A). Second, it appeared to be the only 29-kD protein present in the ECM. In the original guanidine fraction applied to the Q resin, there was a 29-kD species that did not bind to the Q resin and elution of the resin with 1 M salt did not release any other 29-kD proteins (Fig. 4 B). Thus, there were no anionic p29 proteins, all were either neutral or cationic. Furthermore, in the heparin column, no fraction other than the one eluted by 200 mM salt contained a 29-kD protein (Fig. 4 C). Hence, of the cationic 29-kD proteins, all had a similar isoelectric point. A silver-stained gel of the fraction eluted by 200 mM salt from the heparin column showed that the major protein in this fraction is p29 with fainter bands in lower molecular mass regions (Fig. 4 D). Most importantly, p29 purified from high density ECM, when added to DMMA-treated ECM (i.e., p29-depleted ECM), restored its ability to induce apical endocytosis in low density cells (Fig. 2, last bar).

Mass spectrometric fingerprinting of the purified p29 identified it as galectin 3 (see Online Supplemental Materials). Rabbit galectin 3 cDNA (supplied by A. Legrand, Centre National de la Recherche Scientifique, Orleans, France) was cloned into an expression vector, and the his-tagged fusion protein was purified and used for biochemical studies and for the generation of polyclonal antisera in guinea pigs (Fig. 3 B).

Galectin 3 Is Secreted Into the ECM by High Density, but Not Low Density, Cells

The intercalated cell exists in the kidney in a spectrum of types. At one extreme, the β phenotype, similar to the low density phenotype of our in vitro cells, has no apical endocytosis and secretes HCO_3. On the other hand, the α phenotype, similar to our high density form, has vigorous apical endocytosis, with apical H^+-ATPase and basolateral band 3. Remarkably, Winyard et al. demonstrated that galectin 3 is expressed in only one cell type of the adult kidney, the α-intercalated cells (Winyard et al., 1997). These results provide a pleasing congruance between independent investigations. Using cell fractionation, we found that galectin 3 was present only in the cytosol of low density cells, with little if any secretion into the media (Fig. 3 C). However, in high density cells, galectin 3 was present in the cytosol and some was secreted into apical and basolateral media (Fig. 3 C). The media of high density cells were also separated on sucrose density gradients, and we found that they contained soluble multimeric hensin (Fig. 3 D), as reported previously (Hikita et al., 1999). However, galectin 3 was present only in the top of the gradient, rather than with the multimeric hensin.

Using immunocytochemistry of unpermeabilized cells, we found that galectin 3 was present in the ECM of the high density phenotype, but not the low density cells (Fig. 5). There was extensive colocalization of hensin and galectin 3 in unpermeabilized cells after 3 d and 7 d of seeding at high density. Permeabilized low density intercalated cells expressed some galectin 3, but the high density cells had a much higher level of cytoplasmic expression (data not shown). These results confirm our finding that p29 could not be cross-linked to hensin in the media of high density cells. Perhaps the amount of galectin 3 is too small. But the important conclusion of this study is that polymerization of hensin can occur in the absence of galectin 3 in the media of high density cells.

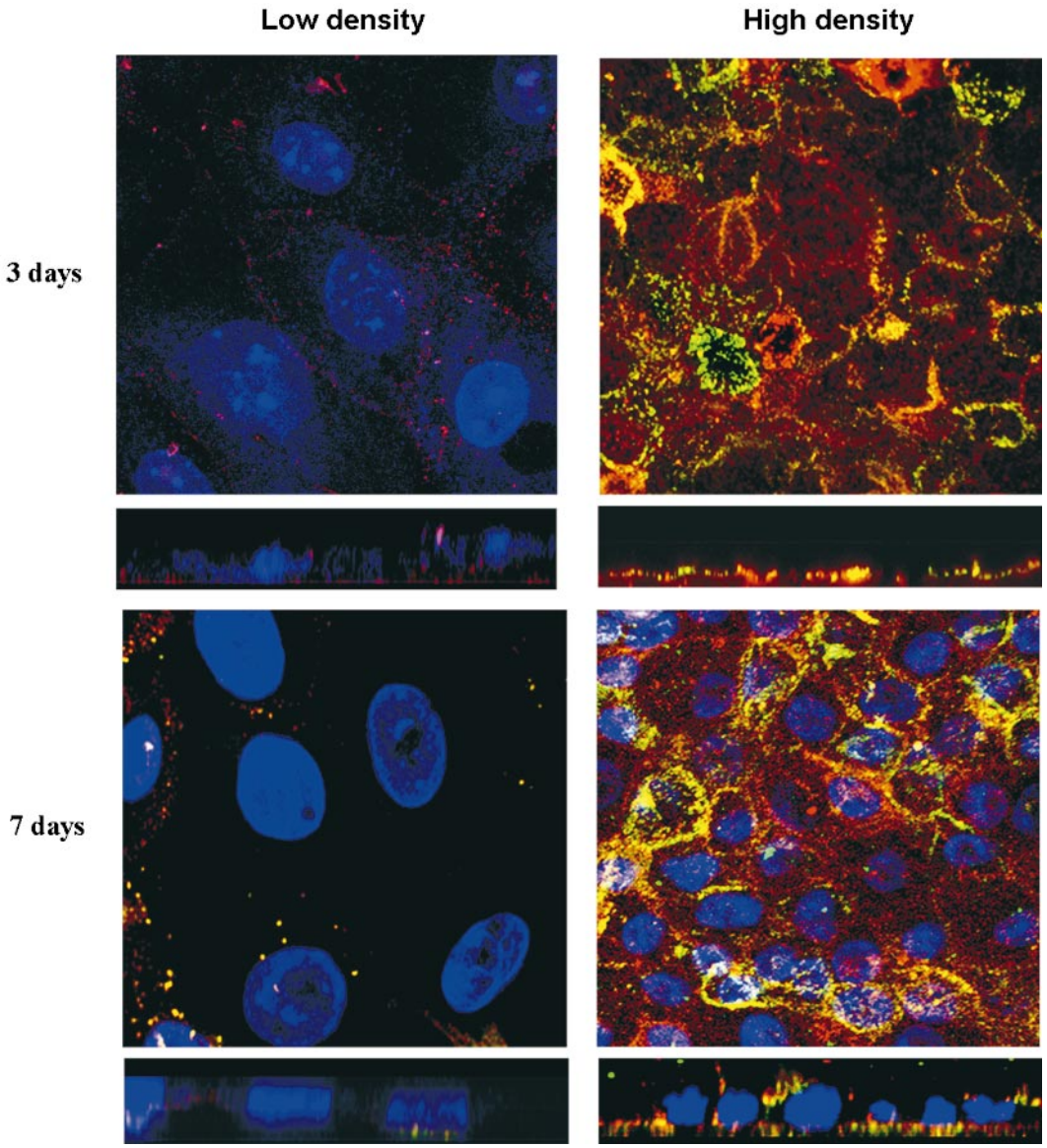

Figure 5. Immunocytochemistry of galectin 3. Cells seeded at low or high density, for the indicated times, were incubated with rabbit anti-galectin 3 and guinea pig anti-hensin antibodies added to both apical and basolateral media at 4°C for 2 h without permeabilization. After they were washed, they were fixed and treated with secondary antibodies. (3 days) Cells were cultured for 3 d and treated as above. The secondary antibodies used were fluorescein-labeled anti–rabbit and rhodamine-labeled anti–guinea pig antibodies. In the low density cells, nuclei were stained with Hoechest staining, which was visualized in the green channel, but was depicted in blue. (7 days) cells were cultured for 7 d and treated as above. The secondary antibodies were Cy5-treated anti–rabbit and rhodamine-treated anti–guinea pig antibodies. Nuclei were stained with Sytox Green. The red channel is depicted in red, the green channel in blue, and the Cy5 is depicted in green.

Galectin 3 Binds and Polymerizes Hensin

Galectin 3 is a lectin that binds to β-galactosides, but unlike other galectins, it also contains an additional domain that mediates homodimerization. We showed previously that monomeric hensin was inactive, and only higher order polymers of hensin were capable of inducing terminal differentiation (Hikita et al., 1999). To test whether galectin 3 can bind hensin, we incubated monomeric hensin (prepared from media of low density cells by sucrose density gradient centrifugation) with excess recombinant his-tagged galectin 3 and precipitated the complex with Ni beads. Fig. 6 A shows that all monomeric hensin bound the Ni resin. Similarly, when soluble hensin dimers and tetramers were prepared by sucrose density gradient centrifugation from the media of high density cells, these too bound to galectin 3. The complex of hensin and galectin 3 was large enough that it was precipitated by centrifugation at 10,000 *g* for 20 min, suggesting that this complex contained several copies of each of its components (Fig. 6 B). To obtain more quantitative information, we added an excess of galectin 3 to monomeric hensin (obtained from low density media) and separated the complex on metrizamide gradients. The apparent molecular mass of the complex increased from that compatible with a monomer of hensin to

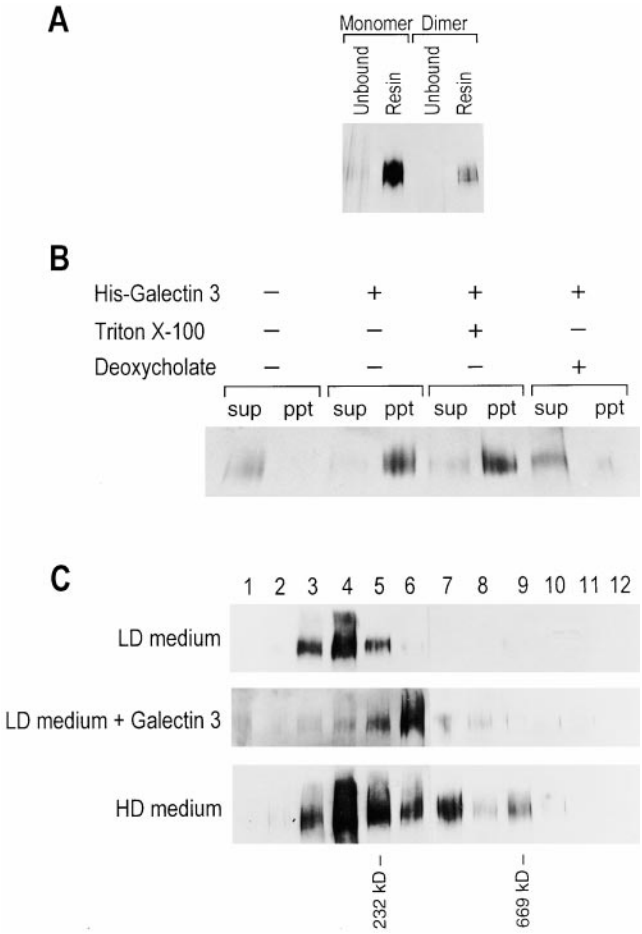

Figure 6. Galectin 3 binds to hensin. (A) Monomeric and dimeric hensin bind to galectin 3. It was previously shown that the media of high density cells contain monomeric and dimeric hensin. Media were collected and concentrated and separated on sucrose density gradients, as described previously (Hikita et al., 1999). The monomeric and dimeric fractions were dialyzed to remove sucrose and then incubated with beads containing his-tagged galectin 3 for 30 min. They were centrifuged and the supernatant and pellets were analyzed by immunoblots using anti-hensin antibodies. (B) His-tagged galectin 3 binds to hensin to form multimers. His-tagged galectin 3 was added to fractions containing hensin (and the described detergents) for 30 min. The combined mixture was centrifuged at 10,000 *g*, and the supernatants and pellets were analyzed by immunoblots using anti-hensin antibodies. (C) Effect of galectin 3 on migration of monomeric hensin in density gradients. Media from low density cells (LD) were mixed with galectin 3 and centrifuged on metrizamide gradients. High density media are shown for comparison. Fractions were precipitated and subjected to immunoblotting with anti-hensin antibodies.

that compatible with a dimer or no more than a tetramer (Fig. 6 C). We point out that hensin extracted from the ECM migrated at the bottom of the gradient, and its molecular mass could not be reduced by treatment with sulfhydryl-reducing agents to any significant degree (Hikita et al., 1999). These results emphasize that the interaction of hensin and galectin 3, though critical for its function and localization, is only a part of the mechanism by which hensin is localized in the ECM.

The interaction of hensin and galectin 3 was disrupted by deoxycholate, but not by Triton X-100, suggesting that some hydrophobic interactions occurred between the two proteins (Fig. 6 B). Remarkably, addition of galectin 3 to monomeric hensin obtained from low density media did not result in a high order polymer (Fig. 6 C). The migration of monomeric hensin in metrizamide gradients slowed down to an apparent density compatible with only a dimer. On the other hand, soluble hensin from high density media migrated at higher densities compatible with tetramers. These results demonstrate that the high density state polymerizes hensin first by an as yet unknown process and that galectin 3 is necessary for an additional process of polymerization (see Discussion).

Purified p29 from high density ECM bound to lactose-agarose beads, confirming that it is galectin 3. However, the binding of hensin to galectin 3 was not disrupted by high concentrations of lactose (250 mM), sucrose (500 mM), or mannose (500 mM). The binding of lectins to glycoproteins is likely mediated by very specific sugar structures whose affinity might be several orders of magnitude higher than that for lactose, sucrose, or mannose. However, at present, we cannot exclude that the binding of galectin 3 to hensin is mediated by protein–protein interaction.

Galectin 3 Is Necessary for the Activity of Hensin

The potency of hensin to induce apical endocytosis is retained during its purification until the last step. When the penultimate fractions (those of the Q resin chromatography) were studied, we found that they contained galectin 3 in fractions that were not compatible with galectin's isoelectric point. Galectin 3 is a basic protein that does not bind to the Q resin (Fig. 4 B). However, the hensin fractions that bound to the Q resin also contained galectin 3 (Fig. 4 E). We had shown previously that these fractions, which we now demonstrate contain only hensin and galectin 3, were active in inducing apical endocytosis (van Adelsberg et al., 1994). DMMA treatment of these fractions released galectin 3 from hensin (data not shown). This purified hensin was adsorbed onto filters and cells were then seeded at low density. After 1 wk of growth, we assayed for apical endocytosis. The highly purified hensin induced a low level of apical endocytosis (2.86 ± 0.7 nl/10 min), but the addition of hensin and galectin 3 together produced a significantly higher rate (4.6 ± 0.8). These endocytosis results are much lower than those presented in Fig. 2, probably because the proteins are adsorbed to filters and subjected to numerous washings to ensure sterility, which likely reduced the total amount of proteins that remained on the filters.

To test whether galectin 3 alone can induce apical endocytosis, we adsorbed purified ECM galectin 3 to filters and seeded cells at low density. There was some apical endocytosis (2.8 ± 0.7). Unfortunately, these results were somewhat marred by our finding that the use of purified ECM galectin 3 often resulted in leaky monolayers, probably because of a factor that was eluted during the purification scheme. (The use of recombinant galectin 3 uniformly resulted in leaky monolayers). We developed a morphological assay of endocytosis using FITC-dextran internalization. Fig. 7 shows that high density cells had a high level of apical endocytosis, whereas low density cells had minimal levels, confirming the utility of this assay. Cells seeded at low density on galectin 3 alone internalized apically

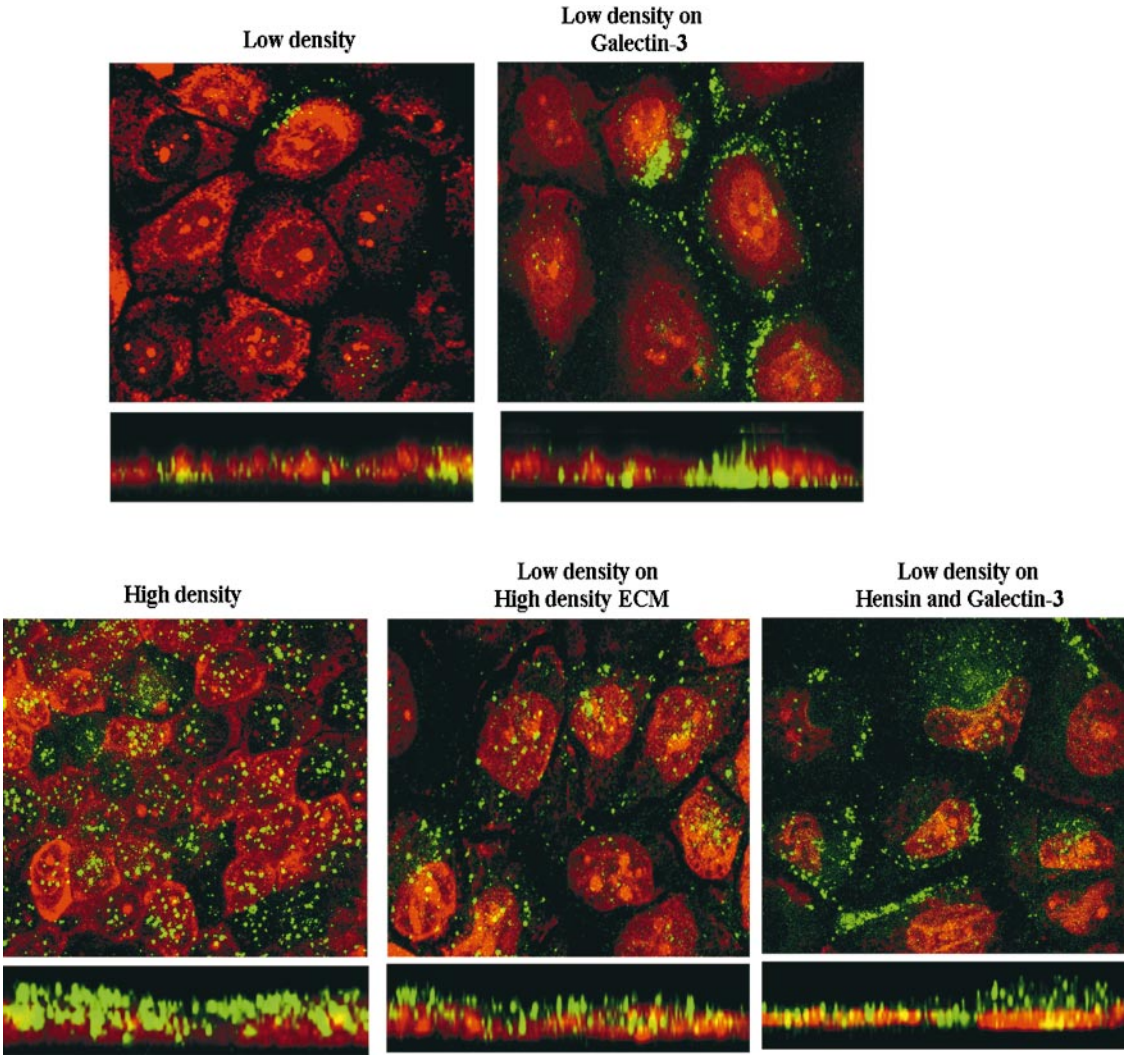

Figure 7. Apical endocytosis using FITC-dextran. Epithelial monolayers were incubated with FITC-dextran for 10 min and then fixed and stained with propidium iodide. Confocal images were obtained. High density cells had vigorous apical endocytosis, whereas low density cells had none. Seeding cells at low density on high density ECM induces apical endocytosis. Cells seeded at low density on purified galectin 3 had no apical endocytosis. Note that in these studies several tight junctions were open, and the FITC-dextran was internalized from the lateral or basal surface. Apical endocytosis was induced in low density cells seeded on filters coated with purified hensin that contained galectin 3 (fractions seen in Fig. 3, B and E). Fields were chosen to demonstrate both the open tight junctions and the presence of apical endocytosis.

added FITC-dextran from the lateral surface of cells that did not form a tight monolayer, but the regions of the monolayer that appeared tight had no apical endocytosis (Fig. 7). Purified fractions that contained both hensin and galectin 3 were able to induce apical endocytosis, whereas those that contained galectin 3 alone did not (Fig. 7). These results demonstrate that a complex of hensin and galectin 3 is needed to induce the change in phenotype. Note that in Fig. 7, the X-Z section was composed from the whole of the X-Y plane, rather than from the 1-μm-thick optical section, as is usually the case. This was done to maximize both the apical and the lateral endocytosis.

We prepared several polyclonal antisera against hensin or galectin 3. Antisera to hensin prevented the induction of apical endocytosis, the appearance of subapical actin cytoskeleton, the induction of subapical cytokeratin 19, the appearance of subapical villin, and columnarization of the cells (Vijayakumar et al., 1999). Antisera to hensin also significantly reduced the induction of apical endocytosis in low density cells seeded on filters coated with hensin and galectin 3. However, two independently produced polyclonal antisera raised to the full-length galectin 3 had no effect on apical endocytosis (data not shown).

Hensin and Galectin 3 Are also Expressed in Other Epithelia

Hensin, or other alternately spliced products from the same gene, are widely expressed in a variety of epithelia, and in the brain and lung macrophages (Takito et al., 1999). Galectin 3 is also widely expressed in epithelia, glia, and other cell types. We found that in adult tissues, which exhibited a continuous level of differentiation, e.g., the intestine or prostate, the pattern of expression of hensin resembles that of intercalated cells (Vijayakumar et al., 1999). In the protoepithelial cell of the intestinal crypts, hensin was expressed in intracellular vesicles and did not localize to the ECM, which is similar to its staining pattern in low density

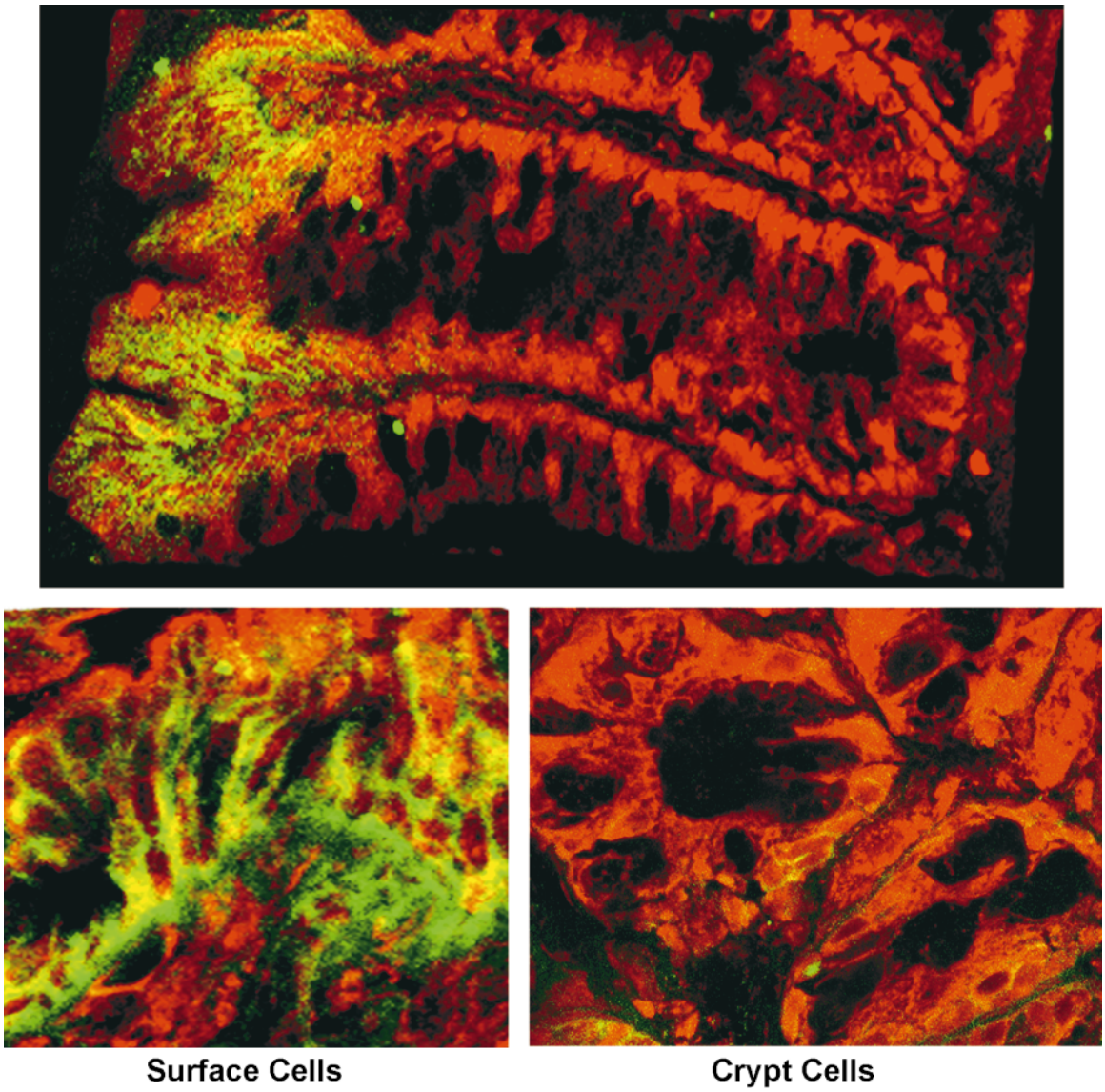

Figure 8. Expression of hensin and galectin 3 in colon. (A) Rat colon sections were stained for hensin (red) and galectin 3 (green). The lower images were taken at higher magnifications. In the surface cells, hensin and galectin 3 surround the cell in a manner suggestive of ECM staining. However, in the crypt, the hensin staining is more diffuse, suggesting intracellular staining. Note the virtual absence of galectin 3 staining in the crypts.

cells. In absorptive villus cells, which are terminally differentiated, hensin was present largely in the ECM. In the colon (Fig. 8) we found that the pattern of expression of hensin and galectin 3 resembles that of the intercalated cell in vitro. In the surface cells (Fig. 8, bottom left), hensin and galectin 3 surround the cells in a pattern that resembles that of ECM staining. In the crypt (Fig. 8, bottom right), hensin is diffuse, suggesting intracellular localization. The crypts contain little or no galectin 3. These studies raise the possibility that hensin and galectin 3 might be involved in the terminal differentiation of other epithelia.

Discussion

Multimeric hensin isolated from the ECM induced terminal differentiation of intercalated cells by "outside-in" signaling in a manner similar to the effect of many factors acting on their receptors. Monomeric hensin was soluble and incapable of converting the intercalated cell from one phenotype to another. When the cells were "activated" by high density seeding, they acquired the ability to polymerize exogenously added monomeric hensin, a process reminiscent of "inside-out" signaling. Here, we presented evidence that one mechanism by which hensin could be polymerized and deposited in the ECM is by the secretion of galectin 3 into the ECM.

Galectins

Galectins, a family of proteins that bind galactose β 1-4 *N*-acetylglucosamine, are present in all metazoa thus far examined (Hughes, 1999). They lack a signal sequence, are located in the cytoplasm, and get secreted by a mechanism that

bypasses the classical secretory pathway (Hughes, 1999). In epithelia, galectin 3 can be secreted into the apical compartment of MDCK cells (Lindstedt et al., 1993), but culturing the same cells in a three-dimensional collagen matrix causes them to be secreted from the basal surface (Bao and Hughes, 1995). In macrophages, galectin 3 is intracellular, but activation of the macrophage either by calcium ionophore, phorbol esters, or thioglycollate results in secretion of the lectin to the outside (Sato and Hughes, 1994). Aside from their sugar-binding activity, their function in cellular processes have been ill defined, but they have been associated with a variety of inflammatory, immunologic, and malignant phenomena (Perrillo et al., 1998). Galectin 3 is widely expressed in epithelia and in other tissues. It differs from other galectins by having an additional dimerization domain (Massa et al., 1993). Galectin 3 bound to monomeric as well as to multimeric hensin. It was secreted by high density cells and localized in the ECM. Remarkably, it was found to be expressed in only one cell type in the adult kidney, the α intercalated cell, the equivalent cell type of the high density intercalated cell (Winyard et al., 1997). This concordance is gratifying and suggests that galectin 3 plays a similar role in vivo to its role in vitro. Deletion of galectin 3 seems to result in phenotypically "normal" mice, though there has been no report of a study of the intercalated or other hensin-expressing cells in these mice (Colnot et al., 1998). Perhaps, its function can be mediated by another galectin; there are at least 10 galectins and several tissues express more than one. In support of this suggestion is the finding that previous studies have suggested that galectin 1 is critical for implantation, but embryos deleted in galectins 1 and 3 are normally implanted, though they express galectin 5 (Colnot et al., 1998). Hence, the diversity of galectins ensures the continuous presence of lectins with at least similar lectin properties, but with other more individual functions, as well.

Outside-In Signaling

Originally discovered as Mac2 in macrophages, galectin 3's binding partner (Mac2BP) was later identified as a protein that contains an SRCR domain (Koths et al., 1993). Given that hensin has eight SRCR domains, we suggest that hensin is "bundled" into a fiber by galectin 3. Preliminary studies of purified hensin–galectin complexes shows images of fibers by negative-staining electron microscopy. The complex formation of hensin with galectin provides a hypothesis for the mechanism of "outside-in" signaling. Many cell surface receptors, such as receptor tyrosine kinases, integrins, or even cadherins, require clustering before a signal can be transmitted into the cell. The SRCR domains of hensin are separated from each other by proline-rich intervening sequences, thus, the binding of soluble monomeric hensin to its receptor is probably monovalent, since the presence of many prolines will likely increase the number of available conformations. It is likely that the hensin–galectin polymers are rigid fibers. Therefore, the eight SRCR domains might then assume a specific conformation presenting closely spaced binding surfaces to the plasma membrane. These might allow receptors to cluster causing signaling. As we demonstrated previously, treatments that dissociated the hensin multimers (e.g., sulfhydryl reducing agents, DMMA) also disrupted its ability to activate the cell, probably because the rigid alignment of the hensin domains is critical for its ability to bind to more than one receptor molecule (Hikita et al., 1999). We speculate that this multivalent binding is the mechanism that causes clustering of the hensin receptors activating a signaling cascade.

Inside-Out Signaling

Resting platelets are unable to aggregate because their integrin receptors have a low affinity for fibrinogen. But following activation by specific ligands, such as ADP, the affinity of their receptors for fibrinogen increases dramatically and aggregation occurs (Shattil and Ginsberg, 1997). This affinity modulation apparently results from binding of signaling proteins to the cytoplasmic domains of the receptor, causing a conformational change in the receptor, and is termed inside-out signaling (Hughes and Pfaff, 1998). One consequence of the increased affinity is a change in the solubility of fibrinogen. Similarly, affinity modulation of α5β1 integrin induces a conformational change in fibronectin that will allow its assembly into fibrils (Schwarzbauer and Sechler, 1999). Although we do not have any information regarding the affinity of the hensin receptor, here, we showed a new mechanism of inside-out signaling. When hensin multimers were isolated from the media of high density cells and added to low density cells, they failed to induce the change in phenotype (Hikita et al., 1999). We showed that these multimers do not contain galectin 3. Only hensin in the ECM, i.e., hensin that is a high order multimer bundled with galectin, produced the largest effect. Removal of galectin from this form of hensin, reduced its activity drastically. We suggest that the process of conversion of hensin from an inactive soluble form to an active insoluble form requires secretion of galectin 3 that will form bundles of hensin.

Inside-out signaling is likely to be a complex process that involves not only affinity modulation and galectin 3 secretion, but also additional events that prepare hensin for deposition in the ECM in a functional form. We found that soluble multimers of hensin, when treated with DMMA, dissociate to monomers, but removal of DMMA resulted in the reassembly of the multimers (Hikita et al., 1999). These results demonstrate that hensin has been permanently altered by the "surface" of the activated cells. When soluble multimers were cross-linked, galectin 3 was not detected, suggesting that the multimerization was not due to the action of the lectin, but rather a conformational change must have occurred that allowed hensin to multimerize. Furthermore, when exogenous soluble monomeric hensin was added to high density cells, it was converted to dimers and higher order multimers, indicating that an extracellular component of activated epithelial cells is capable of inducing multimerization of hensin (Hikita et al., 1999). At present, the mechanism of formation of the soluble multimers by activated cells is unknown. These studies suggest that the hensin pathway might be a useful model to study the complex processes of inside-out signaling.

We are grateful to Theresa Swayne for help in microscopy.

This work was supported by a grant from the National Institutes of Health (NIH), DK20999. The Columbia Confocal Facility is supported by NIH grants RR10506, and the sequencing facility was supported by the National Cancer Center grant P30 CA08748.

Submitted: 24 May 2000
Revised: 29 September 2000
Accepted: 5 October 2000

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