Published in final edited form as:

Anat Rec. 1999 March ; 254(3): 348–359.

ECM-Stimulated Actin Bundle Formation in Embryonic Corneal Epithelia is Tyrosine Phosphorylation Dependent

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Abstract

Previous studies demonstrated that corneal epithelial cells isolated without basal lamina respond to extracellular matrix (ECM) in an actin dependent manner; the basal cell surface flattens and the actin cortical mat reorganizes. We hypothesize that the actin reorganization is initiated by intracellular signaling mechanisms that includes tyrosine phosphorylation and activation of the Rho, MAP kinase, and PI₃ kinase signal transduction pathways. Our goals were to develop a morphological assay to test this hypothesis by answering the following questions: 1) Do the actin bundle formations in the cortical mat have the same configuration in response to different ECM molecules? 2) What is the minimum time ECM molecules need to be in contact with the tissue for the actin to reorganize? 3) Will blocking tyrosine phosphorylation inhibit reorganization of the actin? 4) Are known signal transduction proteins phosphorylated in response to soluble matrix molecules? The actin cortical mat demonstrated distinct bundle configurations in the presence of different ECM molecules. Soluble fibronectin accumulated at the basal cell surfaces 75-fold over 30 min in a clustered pattern. The cells need contact with ECM for a minimum of 10 min to reform the actin bundles at 2 hr. In contrast, two substances that bind to heptahelical receptors to stimulate the Rho pathway, bombesin and lysophosphatidic acid, reorganized the actin bundles in 15–30 min. Focal adhesion kinase, p190 Rho-GAP, tensin, and paxillin were tyrosine phosphorylated in response to soluble fibronectin, type I collagen, or laminin 1. Erk-1, erk-2, and PI₃ kinase were activated after 1 hr stimulation by type I collagen. Herbimycin A blocked actin reorganization induced by ECM molecules. In conclusion, we have developed two morphological assays to examine the response of corneal epithelial cells to ECM molecules. In addition, actin bundle reorganization involved tyrosine phosphorylation, MAP kinase, and PI₃ kinase activation. *Anat Rec* 254:348–359, 1999.

Keywords

actin bundles; extracellular matrix; corneal epithelia; bombesin; lysophosphatidic acid; tyrosine phosphorylation

The focus on cell-matrix receptor molecules, mainly integrins, have contributed greatly to the understanding of cell-matrix interactions (Hynes, 1992). It is now commonly accepted that these molecules not only provide a link between the matrix and cytoskeleton, but also play a

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role in signal transduction (Gumbiner and Yamada, 1993; Sastry and Horwitz, 1993; Schaller and Parsons, 1994; Parsons, 1996; Giancotti, 1997; Hall, 1998).

Many cell culture systems have been developed to study cellular responses to extracellular matrix (ECM). Investigators have examined the immediate responses following first contact of suspended cells, cell spreading (Ingber et al., 1990; Schwartz, 1992; Schwartz and Ingber, 1995), and ECM-coated bead endocytosis (Grinnell and Geiger, 1986; Miyamoto et al., 1995a,b). In contrast, we use a whole sheet of primary epithelial tissue isolated without basal lamina (–BL). These epithelial sheets respond to purified soluble ECM molecules by reorganizing the actin cytoskeleton (Sugrue and Hay, 1981, 1986, 1987; Svoboda, 1992; Khoory et al., 1993). This unique whole tissue model allows the examination of tissue responses to specific molecules without the cellular attachment and spreading behavior observed in cells grown in traditional tissue culture (Burridge, 1986; Burridge et al., 1988, 1990, 1997; Bockholt and Burridge 1995; Burridge and Chrzanowska-Wodnicka 1996). This combined data supports the hypothesis that ECM molecules initiate a signaling cascade. The current understanding of the cascade of events appears to be: ECM proteins bind integrin molecules; FAK may be recruited to the integrin cytoplasmic domain by talin. Talin binds vinculin and paxillin. FAK goes through a conformational change to bind the $\beta 1$ cytoplasmic tail and autophosphorylates after the integrins have clustered via a Rho-mediated mechanism (Giancotti, 1997; Hall, 1998).

The exact order of signaling cascade events is currently being investigated by several laboratories. Schaller and Parsons (1994) have shown that phosphorylation of paxillin creates binding sites for the SH2 domains of several signaling molecules such as Csk and Crk. They hypothesize that paxillin may play a role in activation of GTP binding proteins at specific sites within the cell and that these GTP binding proteins transmit signals from the cell surface to the nucleus and cytoskeleton (Schaller et al., 1995; Schaller and Parsons, 1995). Ridley and Hall (1994) have used a serum starved Swiss 3T3 fibroblast model to demonstrate that the small GTP binding protein, Rho, mediates formation of focal adhesions and stress fibers (Ridley et al., 1993). Serum-starved Swiss 3T3 fibroblasts also respond to lysophosphatidic acid (LPA) and bombesin indicating that these structures form as a result of Rho dependent signaling (Ridley and Hall, 1994). Recently this group has identified the roles of Rho, Rac, and Cdc42 GTPases in the formation of stress fibers in the tissue culture model (Nobes and Hall, 1995; Hall, 1998).

Our whole tissue model supports the hypothesis that the cells respond to ECM molecules by reorganizing the actin cytoskeleton and basal cell surface (Sugrue and Hay, 1981; Svoboda and Hay, 1987; Khoory et al., 1993). At the level of transcription/translation, increased collagen synthesis occurs within 20–24 hr of ECM stimulation. (Sugrue and Hay, 1986; Svoboda and Hay, 1987). It was also shown that laminin and collagen stimulated actin reorganization did not require new protein synthesis, however, the response to fibronectin did require newly synthesized proteins (Sugrue and Hay, 1982, 1986). The proteins required have not been identified.

A number of intracellular proteins have been identified in the cell-substratum interface including α -actinin, vinculin, talin, paxillin, zyxin, tensin, FAK, FAK-related-nonkinase (FRNK), and other signal transduction proteins (Luna and Hitt, 1992; Gumbiner and Yamada, 1993; Schaller and Parsons, 1993). Again, the exact link between the matrix and actin cytoskeleton has not yet been established (Parsons, 1996; Burridge et al., 1997; Giancotti, 1997; Hall, 1998). We have previously identified several actin associated proteins in the actin cortical mat area of embryonic corneal epithelia; α -actinin, vinculin, talin (Khoory et al., 1993; Hirsch et al., 1994), zyxin, and paxillin (Jirawuthiworavong et al., 1995; Svoboda et al., 1999).

Current research has concentrated on the extracellular interactions of various ligands with integrin receptors. However, much less is known about the interactions occurring intracellularly to form the actin rearrangement in whole tissues (Furukawa and Fechheimer, 1997). Therefore, studying the intracellular pathways downstream of integrin ligand interaction is critical to understanding their functions as signal regulators and modulators of actin bundle organization. Characterization of these signal transduction pathways and how they interact with each other are the next pieces to the integrin-mediated signaling puzzle to be solved. The signaling molecules (FAK, PI₃ kinase, MAP kinase, Rho, and p190 Rho-GAP) and their interactions have been studied extensively in several different fibroblast models. However, little is known about their interactions in a whole epithelial tissue model.

Our first goal was to develop reliable assays to establish that whole epithelial sheets respond to specific ECM molecules. We also designed experiments to determine the minimum time the epithelia had to be stimulated with FN or COL to respond by reorganizing the actin microfilament bundles in 2 hr. The next goal was to determine signaling proteins that were tyrosine phosphorylated. We also asked, could ECM stimulated reorganization of the actin cortical mat be blocked with the phosphorylation inhibitor herbimycin A, i.e., is actin reorganization tyrosine phosphorylation dependent? These experiments will establish the model system for future experiments to determine the specific sequence of signaling pathways in an intact epithelia.

Materials and Methods

Tissue Isolation

Fertile white leghorn chicken eggs (SPAFAS, Inc., Norwich, CT), were incubated for 6–8 days at 39°C. The embryos (Hamburger-Hamilton stages 27–34) were removed from the eggs and rinsed in Hank's balanced saline solution (HBSS; GIBCO Laboratories, Grand Island Biological Co., Grand Island, NY). The animals were handled in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Whole corneas, with some surrounding sclera, were removed from the embryos with fine forceps. Corneal epithelia isolated without the basal lamina (–BL), were treated for 2–4 min at 37°C in 0.8 mg/ml trypsin and 0.8 mg/ml collagenase (Sigma, St. Louis, MO) in calcium-magnesium-free HBSS. After the enzyme treatment, the corneas were rinsed in Ham's F12 medium (GIBCO Laboratories) and then HBSS to stop the action of the enzyme. A continuous sheet of corneal epithelial cells was dissected from the underlying stroma with fine tweezers and placed basal-side down on a black polycarbonate filter (3 mm diameter, 0.4-μm pore size; Poretics, Livermore, CA).

Organ Culture

Epithelial sheets were isolated without the basal lamina (–BL) using trypsin and collagenase (Svoboda and Hay, 1987; Svoboda, 1992) and placed on black polycarbonate filters (Fig. 1). The epithelial cells were suspended at the air-media interface by a triangular-shaped wire grid in an organ culture dish (Becton Dickinson Labware, Franklin Lakes, NJ). The medium height barely covered the apical surface of the tissue. Cultures were incubated at 37°C in a humidified gas mixture (5% CO₂ and 95% air). The control culture medium was composed of Ham's F12 medium (GIBCO), antibiotic/antimycotic (GIBCO), and 50 μm ascorbic acid (Sigma). Epithelia were incubated in control media with stimulating molecules for various times (0–2 hr). The ECM molecules used were 50 μg/ml human plasma fibronectin (FN, Sigma), 100 μg/ml Bovine type I Collagen (COL, Collaborative Research, Bedford, MA), 10 μg/ml laminin (LN, Collaborative Research), 12 nM bombesin (Sigma), or 10 ng/ml lysophosphatidic acid

(LPA, Sigma). After various incubation times with stimulating molecules (0–2 hr), the epithelia were fixed and stained with phalloidin to determine if the actin cortical mat reorganized.

To test the minimum time needed to expose epithelial tissue to ECM molecules the epithelia were cultured in the presence of an ECM protein (COL or FN) for different experimental times (5, 10, 15, 30 min). The ECM media was removed and the epithelia then incubated with control media for a total culture time of 2 hr.

Some epithelia were incubated in the presence of FITC-labeled FN or COL for different times, then fixed and viewed on the confocal microscope. Additional epithelia were counter stained with rhodamine phalloidin to assess the organization of the F-actin.

Actin Labeling

The F-actin in the cells was labeled with fluorescently-tagged phalloidin (Molecular Probes, Inc., Eugene, OR; Svoboda, 1992). The epithelia were incubated with FITC-phalloidin (1:20 dilution of phalloidin stock solution in 3.7% formaldehyde, 0.01% lysopalmitate) for 30 min at RT, followed by three 10-min rinses in PBS. The fluorescent phalloitoxins were kept as stock solutions containing 300 units/3 ml methanol (approximately 3.3 μ M). A unit is defined as the amount of material needed to stain one microscope slide of fixed cells.

Confocal Laser Scanning Microscopy

Specimens were analyzed with the Leica upright CLSM, equipped with an argon ion laser. The continuously variable detection pinhole was set at the minimum size for optimal signal (Pawley, 1990; Wilson, 1990). The typical *z*-series was composed of optical sections in the *xy* optical plane. These images were *en face* optical sections through the vertical axis of the tissue. Specimens were also scanned in the *xz* plane, allowing the tissues to be visualized in cross section. The point-spread function for the confocal microscope is greater in the *xz* than the *xy* focal plane (Pawley, 1990), causing the images to be more distorted.

Images were analyzed, enhanced, and stored on an optical disc. Black and white and color photographs were computer generated with minimal computer enhancement. These images were printed directly to a Kodak 8650 dye sublimation printer from Photoshop or Pagemaker (Adobe) documents.

Labeling ECM Molecules

Fluorescein isothiocyanate (10 mg/ml in DMSO, Sigma) was coupled to purified protein in 0.1 M carbonate-bicarbonate buffer (pH 9.0) overnight at 4°C. FN (1 mg/ml, Collaborative Research) and type I rat tail COL (1 mg/ml, Collaborative Research) were used. The excess isothiocyanate was removed by exhaustive dialyzing against Ham's F12 culture media in a sterile container. The ratio of dye to protein was measured with a spectrophotometer and the biological activity of the protein was assessed in the morphological assay. The FITC-labeled FN and COL were stored at 4°C until needed for experiments.

The intensity of FITC labeled molecules was determined utilizing NIH Image. Epithelia (*n* = 3/treatment group) were scanned in the *xz* optical plane then transferred to a power Macintosh computer. The mean intensity was calculated from a line that intersected the brightest points through the basal region of each epithelia (Fig. 2, arrowheads). The mean intensity was standardized to the background of each sample. Intensity values were set to 0 = black (minimum) to 255 = white (maximum).

Western Blot Analysis

Epithelia were isolated and cultured as described above, then placed in sample buffer with 1 mM sodium orthovanadate, boiled for 5 min, and resolved on either 15 or 7.5% SDS-PAGE under reducing conditions (Laemmli, 1970). The proteins were transferred to Immobilon PVDF membrane (Millipore Corporation, Bedford, MA). Affinity purified anti-phosphotyrosine (PY20, anti-P-Tyr), paxillin (PAX), pp125^{FAK}, MEK, erk-1/2, and PI₃ kinase antibodies were commercially obtained (Transduction Laboratories, Lexington, KY). MAP kinase activity antibody was obtained commercially to detect phosphorylation of erk-1 and erk-2 (Promega, Madison, WI). Detection of bound primary antibodies was determined by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG and subsequent enhancement with chemiluminescence (Dupont NEN, Boston, MA).

Phosphorylation Inhibitor Experiments

Epithelia were isolated and cultured as described above, treatment groups included control media with one of the following combinations: 1. (Control/no treatment [NT]) 0.01% dimethyl sulfoxide (DMSO, Sigma); 2. Soluble ECM molecule (10 µg/ml laminin [LN], Collaborative Research; 50 µg/ml human plasma fibronectin [FN], Sigma; or 100 µg/ml Bovine type I Collagen [COL], Collaborative Research) and 0.01% DMSO; and 3. Soluble ECM molecule (LN, FN, or COL) and 0.17, 1.7, or 5 µg/ml Herbimycin A (Kamiya Biological Co., Thousand Oaks, CA). Herbimycin A was kept in DMSO stock solutions at 4°C.

The actin morphological assay was used to determine if the ECM stimulated actin cortical mat reorganized in the presence of each concentration of herbimycin A (HA). Controls for these experiments were parallel cultures incubated in the presence of ECM molecules without inhibitor and cultures incubated with the carrier alone (DMSO). After a two hr incubation, the epithelia were fixed and stained with phalloidin to determine if the actin cortical mat reorganized.

Organ culture experiments were repeated three times (n = 5–10 epithelia/treatment group). The epithelia were examined immediately after the phalloidin staining was completed. Evaluation of the actin cortical mat was determined with a Leica CLSM model confocal microscope. Each group of epithelia (n = 5–8) was scored (+ACM, ±ACM or –ACM). The percentage of epithelia + and –ACM per inhibitor dose was recorded and graphed. Confocal scans were taken from a representative epithelia/treatment group. Black and white or pseudocolour images were computer generated with a minimum of computer enhancement and arranged with Adobe Photoshop and Pagemaker then printed on a Kodak 8650 dye sublimation printer. All controls were collected, enhanced, and photographed with the same conditions as the positive tissue.

Results

The actin cortical mat has varied morphology in the presence of different ECM substrates

F-actin (labeled with FITC-phalloidin) distribution demonstrated distinct organizational patterns when the epithelia were cultured for 2 hr in the presence of different ECM molecules or direct Rho stimulators (Fig. 1). Epithelia isolated –BL have large cytoplasmic extensions along the basal cell surface that contain F-actin (Fig. 1A,B; Sugrue and Hay, 1981; Svoboda, 1992). A single optical section taken *en face* (xy optical plane, at the level of the dashed line on Fig. 1A) demonstrates that the most intense actin label is localized to bright spots that represent the basal cytoplasmic blebs (Fig. 1B, arrowheads). This single optical section was scanned slightly tangentially to visualize the basal region of the sheet of epithelial cells. In contrast, epithelia cultured in the presence of type I collagen have large F-actin bundles in the actin cortical mat region (Fig. 1C, dashed line) that align from cell to cell for 80 to 100 µm (Fig. 1D, arrowheads). We know from previous electron microscopic studies that the actin

bundles are not continuous, but are aligned from cell to cell with an elaborate attachment complex at the basal-lateral membrane (CMAX; Svoboda et al., 1999). The intensity of phalloidin staining increased in the basal region of the epithelial tissue (intensity wedge, black = no signal, white = maximum signal). In the presence of fibronectin (FN), the actin filament bundle pattern (Fig. 1E, arrowheads) contained very short actin filaments that did not align from cell to cell. The F-actin distribution in epithelia cultured in the presence of laminin 1 (Fig. 1F) included both long actin bundles (arrowheads) and short bundles. Interestingly, the neuropeptide and Rho pathway stimulators, bombesin (Fig. 1G), and LPA (Fig. 1H) reorganized the ACM very quickly (15 to 30 min) inducing actin bundles similar to fibronectin or laminin (Fig. 1E,F). The bombesin-stimulated tissue sample was not flat and an optical section of the ACM was scanned at a tangential angle (Fig. 1G, between dashed lines). The images obtained through the other ECM stimulated specimens were through the optical plane depicted with the dashed line in Figure 1C. In summary, different matrix molecules and different Rho pathway stimulators induced distinct actin bundle patterns in the basal actin cortical mat of this whole epithelial tissue as shown with single optical sections. The z axis resolution was less than 0.2 μm , as the pin hole was set to the minimal aperture and a 50 $\times$ water objective lens ($n_a = 1.0$) was used to acquire the data. Furthermore, the presence or absence of varied actin bundle distribution patterns was used as a morphological and biological assay to determine the consequences of experimental conditions.

FITC labeled fibronectin and collagen bound to the basal cell surface

After determining that the FITC label did not affect the ability of the corneal epithelia to reorganize the actin, FITC-labeled FN molecules were incubated with epithelia isolated –BL for specific time intervals (0, 5, 10, 15, and 30 min). The epithelia were rinsed, fixed, and examined using confocal microscopy. FITC-labeled FN bound to the basal cell surface (Fig. 2A–D) and the intensity of FITC label increased 75-fold in 30 min (Fig. 2A–D). Between 0 and 5 min the intensity increased 25-fold, then another 25-fold between 10 and 15 min and 15–30 min (Fig. 2 intensity wedge and graph). This experiment did not measure the dissociation constant as the tissue was fixed before confocal analysis. In addition, the maximum binding was not obtained as the curve appears linear. The most interesting observations were that the FN bound gradually over time and that the staining was not uniform. The brighter spots appear to increase in intensity, indicating that integrin clustering may be increasing in the 30 min observation window. Some epithelia were double-labeled with rhodamine phalloidin to demonstrate that the FITC-labeled FN or COL reorganized the actin (data not shown), indicating that the labeled ECM molecules maintained their biological function. In summary, FN and COL bound to the basal cell surface in a nonuniform pattern over a 15–30 min time period causing actin reorganization within 2 hr. The FITC label did not block reorganization of basal actin during those 2 hr.

The actin cortical mat will reorganize after a short exposure to fibronectin or collagen

After establishing that the FITC-FN and COL bound to the basal surface in 30 min. We also determined if the epithelia were incubated with ECM for short times, then reincubated in control media (no ECM), if the ACM would reorganize after 2 hr. This experiment established that the ECM molecules were actually bound to receptors and initiating the reorganization of ACM. All previous experiments had incubated the epithelia in soluble ECM molecules for a minimum of 2 hr (Sugrue and Hay, 1981, 1982, 1986; Svoboda, 1992). We found that the number of epithelia that reorganized the ACM (designated as +ACM) increased with time in soluble ECM (Fig. 3). If the –BL epithelia were not treated with ECM, their ACM did not reorganize and were designated –ACM according to the morphological assay. However, after a 10 min incubation with FN or COL, the ACM reorganized in 22% of the epithelia. After an additional 5 min of exposure (15 min total exposure), over 80% of the epithelia scored +ACM. FITC-FN binding increased from 5 to 10 min, then increased again with longer exposure (Fig.

2, graph). We concluded that epithelial sheets required interactions with soluble ECM molecules for a minimum of 15 min for ACM reorganization to occur in 2 hr. These two sets of experiments indicated that the ECM molecules bound to the basal cell surface and initiated signaling events necessary for actin cytoskeletal rearrangement by 2 hr.

Tyrosine phosphorylation of known signaling proteins is initiated by COL stimulation of embryonic sheets of epithelia

To investigate the signaling events occurring during ECM binding to the basal surface, Western blot analysis of COL-stimulated epithelia was performed. Lower molecular weight tyrosine phosphorylated proteins (PY20, Fig. 4A) were separated on a 15% SDS-PAGE gel (>130 kDa), whereas higher molecular weight proteins, tensin, and p190 Rho-GAP were separated on 7.5% gels. FAK and paxillin were identified in tandem and parallel Western blots (Fig. 4B) from 7.5% gels. In addition, immunoprecipitation of proteins with phosphorylated tyrosines followed by Western blot analysis of tensin, p190 Rho-GAP, FAK, and paxillin (Fig. 4C) confirmed the Western blot analysis.

Corneal epithelia were isolated –BL and incubated with COL for various time intervals (0–60 min, Fig 4A) and compared to epithelia incubated in ECM-free media (0 min, Fig 4A). Several proteins had tyrosine residues phosphorylated in the control (0 min) epithelia that did not change in response to COL. However, tyrosine phosphorylation of other proteins (125, 85, 70, 44, and 28–30 kDa) changed in response to COL (Fig. 4A). The 15% gel did not allow the higher molecular weight proteins to enter the gel, therefore, 7.5% gels were used to determine if larger proteins were phosphorylated. Tensin migrated as a doublet in the 220 kDa range (Fig. 4B) in 7.5% gels and became tyrosine phosphorylated in the presence of ECM molecules (Fig. 4C,D). Tensin co-immunoprecipitated with FAK and PY20, but did not co-immunoprecipitate as strongly with paxillin (Fig. 4D). p190 Rho-GAP also migrated as a doublet that shifted from its lower to the higher mw form in the presence of COL (Fig. 4B), however, this protein could not be isolated by phosphotyrosine immunoprecipitation, indicating that the epitope may be inaccessible. FAK migrated at ~125 kDa as a doublet (Fig. 4A) that was identified by subsequent Western analysis (Fig. 4B) and co-immunoprecipitation (Fig. 4C). The total protein level of FAK did not appear to change over the time studied (Svoboda et al., 1999), however, the tyrosine phosphorylation levels changed from 0 to 15 min then continued to increase through 60 min (Fig. 4A). A tyrosine phosphorylated 68 kDa protein was identified as paxillin (Fig. 4B,C). COL or FN stimulation increased the phosphorylation of tensin, p190 Rho-GAP, FAK, paxillin, and some low molecular weight proteins. In addition, anti-phosphotyrosine immunoprecipitated FAK, tensin, and paxillin but not p190 Rho-GAP or another control, vinculin. Tensin co-immunoprecipitated with FAK indicating that these proteins were closely associated. Individual protein levels did not appear to change during the 2 hr COL or FN stimulation (Svoboda et al., 1999).

Map Kinase And Pi_3 Kinase Increase Phosphorylation In Col Stimulated Epithelia

Other lower molecular weight proteins increased tyrosine phosphorylation following COL or FN stimulation (Fig. 4A). Several of these proteins occurred in the range of 40–45 kDa, indicating that they may be in the MAP kinase family. It is well known that the MAP kinases can respond to a variety of extracellular signals including osmotic stress, heat shock, cytokines, and mitogens (Kortenjann and Shaw, 1995). Two of the MAP kinases named the *extracellular signal regulated protein kinases* (erk-1 and erk-2) translocate to the nucleus after activation to regulate the expression of various transcription factors such as c-myc, c-fos, and c-jun. Activation of the MAP kinase pathways has been identified as a mechanism that integrins use to regulate gene expression leading to cell shape changes during cell spreading or migration (Robinson and Cobb, 1997). Therefore, as we had seen lower molecular weight proteins

became tyrosine phosphorylated in response to ECM (Fig. 4A), we initiated experiments to determine the identity of these proteins.

The purpose of this study was to characterize further potential signaling molecules downstream of Rho and FAK such as MAP kinase or PI₃ kinase. We examined MAP kinase protein levels (erk-2, Fig. 5A) and MEK-2 (Fig. 5B) and phosphorylation in corneal epithelia isolated –BL and stimulated with COL (Fig. 5C). In addition, PI₃ kinase was analyzed from corneal epithelia isolated –BL and cultured with COL for 2 hr compared to nontreated epithelia (NT; Fig. 5D). The amounts of the 42 kDa protein, erk-2, were constant in control (NT) and COL-stimulated epithelia (Fig. 5A). There was a slight shift in mobility of erk-2 that was consistent with increased phosphorylation after 2 hr of COL stimulation. The same Western blot was reprobed for MEK-2 (Fig. 5B). The 42 kDa protein was identified as erk-2 while the 46 kDa protein was MEK-2 (Fig. 5B). The protein levels of erk-2 and MEK-2 appeared constant in control and COL-stimulated epithelia. However, activated erk-1 and erk-2 (denoted by *, Fig. 5C) were detected with an anti-human antibody that specifically binds phosphorylated erk-1/2 proteins. Although the staining was weak, there was a definite increase in activated erk-1/2. The weak staining may be due to the cross species antibody used in this study. Phosphorylated erk-1 (44 kDa) and erk-2 (42 kDa) increased over time with COL-stimulation (Fig. 5C). The 85 kDa subunit of PI₃ kinase was expressed at low levels in blebbing epithelia isolated –BL (NT, Fig. 5D) but increased following 1 hr of COL-stimulation (Fig. 5D). In summary, PI₃ kinase protein levels and erk-1/2 activation increased in COL stimulated epithelia. These events occurred late compared to p190 Rho-GAP and FAK activation, indicating that they are most likely downstream of FAK mediated signaling events.

Herbimycin A blocked actin cortical mat reformation in a dose dependent manner

The next set of experiments established that the phosphorylation events observed in response to ECM stimulation were necessary for actin reorganization. ACM reformation stimulated by ECM molecules (LN, FN, or COL) was inhibited in a dose dependent manner by herbimycin A (HA; Fig. 5D). The cells appeared to have a normal morphology when examined by transmission electron microscopy (data not shown) and the actin organization throughout the epithelium appeared normal except in the basal ACM region. Sample micrographs of optical sections through the ACM of selected epithelia demonstrate the morphological analysis of the ACM. Epithelia cultured in a low dose (LD) of 0.17 μ M (Fig. 6A), medium dose (MD) 1.7 μ M (Fig. 6B), and high dose (HD) 5 μ M HA (Fig. 6C) demonstrated that the ACM may be present in epithelia exposed to the lowest dose (Fig. 6A) comparable to controls (Fig. 1D). The epithelia scored as $\pm$ ACM contained some organized F-actin with bright punctate dots indicating that the basal cell surface was blebbing (Fig. 6B, arrowheads). The epithelium from the high dose group (Fig. 6C) consistently demonstrated only punctate spots of actin staining (arrowheads) indicating that the basal cell surface was covered with blebs. Epithelia cultured in the presence of COL, LN, or FN elicited similar results (Fig. 5D). Even at low doses, HA significantly decreased the reorganization of the ACM (Fig. 5D) as compared to controls (ECM only).

Treating the epithelia with genistein blocked laminin stimulated reorganization of the actin cortical mat (data not shown). However, the broader phosphorylation inhibitor, staurosporine, did not completely inhibit the reorganization of the actin cortical mat (data not shown; Svoboda et al., 1994). In this set of experiments, we used a staurosporine dose (10^{-6} M) that blocked cell migration in culture (Schwartz et al., 1990). The cells appeared to have a normal morphology when examined by transmission electron microscopy (data not shown) and the actin organization throughout the epithelium appeared normal except in the basal ACM region.

In summary, in this whole tissue model, Herbimycin A blocked ECM stimulated actin reorganization in a dose dependent manner. This effect was observed in the presence of three matrix molecules including laminin 1, human plasma fibronectin, and type I collagen.

Discussion

Actin bundles are formed when actin filaments crosslink into arrays with the long axis of filaments in parallel to each other. Actin bundles form in diverse cell types to serve specific functions (Furukawa and Fechheimer, 1997). This whole-epithelial model differs from other well-characterized actin filament bundles found in the intestinal brush border, stereocilia of the ear, invertebrate sperm, *Drosophila* egg chambers or bristles, and *Dictyostelium* movement (Furukawa and Fechheimer, 1997). Specifically, our epithelial tissue reorganizes the actin cytoarchitecture in response to specific ECM, lipid, and neuropeptide molecules.

The simple epithelial model described has specialized cell-cell and cell-matrix domains that are responsible for stability, differentiation, and communication from the microenvironment. Utilizing this whole corneal epithelial model, we have determined that the reorganization of the basal actin cytoskeleton in response to specific molecules is a tyrosine phosphorylation dependent mechanism involving signaling molecules p190 Rho-GAP, FAK, paxillin, tensin, MAP kinase (erk 1/2), and PI₃ kinase. This is the first study to show that whole epithelial tissue responds to matrix molecules similar to cells cultured *in vitro*.

The first indication that an actin cortical mat (ACM) was present in the corneal epithelium was based on cross-sectional data from electron micrographs (Meier and Hay, 1974; Sugrue and Hay, 1981, 1986, 1987). Although the actin network was visible in many micrographs of the corneal epithelium, it was not present in all. In some micrographs the actin bundles aligned from cell to cell with lateral membranes interrupting the bundles. However, the embedding medium, plastic, hides some cytoskeletal elements as the structures are the same optical density as plastic. A new morphological assay to evaluate the F-actin pattern in the basement membrane zone in whole corneas and the isolated corneal epithelia was developed using phalloidin staining and confocal microscopy (Svoboda, 1992). It was shown that an extensive ACM was present in the basal epithelial compartment and is a dynamic structure that responds to ECM molecules (Sugrue and Hay, 1981, 1986; Svoboda and Hay, 1987; Svoboda, 1992; Khoory et al., 1993). The embryonic corneal epithelium is two cell layers thick composed of a periderm and basal layer. Previous studies have shown that when soluble ECM molecules such as FN, COL, and LN were added to the culture media, the blebs reorganized and reformed the ACM within 2 hr (Sugrue and Hay, 1982). Other extracellular matrix molecules including proteoglycans and hyaluronic acid did not reorganize the actin (Sugrue and Hay, 1981, 1982). If cytochalasin D was added with laminin, the ACM did not reorganize (Svoboda and Hay, 1987). In addition, the blebs did not retract in the presence of CD indicating that this was an actin-dependent mechanism (Svoboda, 1992). Epithelia treated with cyclohexamide to block protein synthesis responded to LN and COL but not FN (Sugrue and Hay, 1982, 1986).

It was previously shown that FITC labeled ECM molecules bound to whole floating epithelial sheets and reorganized the basal cell surface after a 3 hr incubation. Furthermore, the bound ECM could be competitively removed with only similar molecules, indicating a specific receptor subtype was utilized for each ECM molecule class (Sugrue and Hay, 1986). We extended these previous experiments to determine the minimum time the cells required incubation in the presence of soluble ECM molecules to bind to the basal cell surface. This unique model also demonstrates that significantly different actin patterns are induced by different stimulating molecules. The ECM molecules bind to different integrin receptors or other nonintegrin receptors, while bombesin and LPA bind to seven-membrane spanning G-protein linked receptors. The action of neuropeptides including bombesin have recently been

linked to tyrosine phosphorylation of multiple substrates including FAK, p130, and paxillin in Swiss 3T3 cells (Zachary et al., 1993). Tyrosine phosphorylation of FAK and paxillin in fibroblasts may also be induced by bioactive lipids such as lysophosphatidic acid or sphingosylphosphorylcholine (Hall, 1998).

Other actin associated proteins that are also present in the basal epithelial area, including α -actinin (Khoory et al., 1993), vinculin (Zieske et al., 1989; Jirawuthiworavong et al., 1995; Svoboda et al., 1998), talin (Burridge et al., 1988), zyxin (Beckerle, 1986; Jirawuthiworavong and Svoboda, 1995; Svoboda et al., 1999), and FAK (Schaller and Parsons, 1993; Parsons et al., 1994; Svoboda et al., 1999). Recently, our lab has identified a specialized basal lateral domain we have termed the *cell matrix attachment complex* (CMAXs). We have characterized actin-associated proteins including α -actinin, vinculin, zyxin, FAK, paxillin, and tensin at the CMAXs (Svoboda et al., 1999).

The role of tyrosine phosphorylation in actin reorganization

Several early intracellular events that occur when integrin molecules bind to ECM include changes in intracellular pH, Ca^{++} , and the phosphorylation of several proteins including FAK in cultured fibroblasts (Ingber et al., 1990; Schwartz, 1992; Schaller et al., 1995; Wu et al., 1995). In several cell culture systems, it has been shown that cytoskeletal proteins are phosphorylated on tyrosine when concentrated in focal adhesions. In particular, FAK and paxillin become tyrosine phosphorylated at these regions. As demonstrated in this study FAK, paxillin and several other proteins became phosphorylated in response to soluble ECM molecules.

This FN binding study supports the hypothesis that the accumulation of matrix was gradual (over 30 min) and that some areas of the basal surface appeared to bind more FN (Fig. 2), indicating that the integrin receptors may have been clustering. Focal adhesion kinase (FAK) is a 125 kDa protein that is a member of the family of nonreceptor protein tyrosine kinases. Experiments involving integrin clustering show recruitment of FAK to focal adhesions by binding directly to cytoplasmic domains of $\beta 1$ integrin. FAK autophosphorylates and then phosphorylates other substrates such as paxillin. There are also sequences in the carboxyl end of FAK called FAT sequences that target the protein to focal adhesions. These observations suggest that integrin-mediated signaling requires recruitment and direct interaction with FAK for functional formation of focal adhesions (Schaller and Parsons, 1994; Giancotti, 1997).

In this study, herbimycin A blocked the reorganization of the ACM in a dose dependent manner with all substrates tested. This was the first indication that tyrosine phosphorylation was required for actin reassembly in this whole tissue. In other studies, when tyrosine phosphorylation of FAK was inhibited by tyrosine kinase inhibitors such as genistein or Herbimycin A, cells rounded up and lost focal adhesions (Schaller and Parsons, 1993). More recently the role of FAK in the formation of focal adhesions has been questioned (Burridge et al., 1997) as FAK^{-/-} cells don't express the protein, but can form focal adhesions (Ilic et al., 1995). In addition, cells that have decreased levels of functional FAK can still reform focal adhesions (Gilmore and Romer, 1996).

Bombesin and LPA reorganized the ACM four- to fivefold faster than ECM molecules. It has been hypothesized that tyrosine phosphorylation of FAK by bombesin, LPA, and pp60v-src bypasses and perhaps mimics the phosphorylation caused by integrin activation (Rozengurt, 1995). Recently, it was shown that the substrates that stimulate the actin through a hepatelical receptor activates the G-protein, Ga_{13} . Ga_{13} -GTP is a guanine exchange factor (GEF) for Rho, providing a biochemical link between the heterotrimer G-protein and a small GTPase (Hart et al., 1998). Our system actually demonstrates that the activation of the Rho pathway directly

with bombesin or LPA increased actin polymerization and actin/myosin contraction necessary for bleb retraction and actin bundle reformation in less than 30 min.

The MAP kinase and phospholipid signaling pathways are activated by ECM molecules in this tissue. We demonstrated that PI₃ kinase protein levels increased in response to collagen, whereas, MAP kinase protein levels did not increase. In contrast, the activated forms of erk-1/2 did increase slightly in response to collagen stimulation. We suspect that the response is stronger than this data indicates as the antibody was anti-human and we were examining chick tissue. FAK can also interact with the phospholipid signaling pathway via PI₃ kinase. PI₃ kinase phosphorylates PIP (PI[4]phosphate) or PIP₂ (PI[4,5]biphosphate) at the D3 position to generate respectively, PI[3,4]P₂ or PI[3,4,5]P₃ (Clark and Brugge, 1995). These phospholipid byproducts have been implicated in downstream signaling of cytoskeletal reorganization through interactions with profilin, gelsolin and Rac (Divecha and Irvine, 1995). In addition to its phosphorylation properties, PI₃ kinase can also act as a ser/thr protein kinase (Divecha and Irvine, 1995). In NIH 3T3 mouse fibroblasts, FAK was found to be stably associated with PI₃ kinase and tyrosine phosphorylated the p85 subunit of PI₃ kinase *in vitro*. The binding affinity between FAK and PI₃ kinase increased when FAK was autophosphorylated. During cell adhesion, there was increased tyrosine phosphorylation of the p85 subunit of PI₃ kinase (Chen et al., 1994). As MAP kinase activity levels and PI₃ kinase levels change in response to COL-stimulated reorganization, it suggests that there is modulation of MAP kinase and PI₃ kinase activities or protein levels. We hypothesize that the MAP kinase and PI₃ kinase signaling pathways are required for ECM-stimulated ACM reorganization in intact embryonic avian corneal epithelia.

Immunoprecipitation studies confirmed that tensin, FAK, and paxillin were tyrosine phosphorylated in the presence of COL. However, p190 Rho-GAP was not immunoprecipitated by anti-phosphotyrosine antibodies. This confirms previous studies that have shown phosphorylated p190 Rho-GAP binds to RasGAP to form a complex (Hu and Settleman, 1997). We hypothesize that this complex may decrease or slow down the GAP function of these proteins, allowing RhoGTP to stay activated longer. It has been established that the activated Rho increases actin polymerization and actin/myosin contraction that allows more integrin clustering (Hall, 1998). Our binding study supports this hypothesis as the FITC-FN bound in a nonuniform distribution and increased binding over 30 min. During this 30 min, tyrosine phosphorylation of tensin, p190 Rho-GAP, FAK, and paxillin increased. In conclusion our data supports the current signaling hypothesis that ECM binding to integrins initiates a tyrosine phosphorylation dependent pathway. When tyrosine phosphorylation is inhibited, actin reorganization is blocked. We have also established an *in vitro* whole tissue culture system that is responsive to ECM stimulation and can be readily utilized to determine the role of signaling molecules in actin cytoskeletal reorganization. Future studies will concentrate on delineating specific proteins and their interactions in the signaling cascade with the hope of achieving a better understanding of the role of matrix, integrins, and/or other receptor molecules in cellular signaling.

Acknowledgments

Grant sponsor: NIH; Grant numbers: R01EY-08886 and AG055706

We thank Dr. Carl Franzblau, the Associate Dean for Graduate Biomedical Sciences, for his assistance in establishing the Leica Confocal Facilities at Boston University School of Medicine.

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Abbreviations

ACM	actin cortical mat
BL	basal lamina
CD	cytochalasin D
CMAX	cell matrix attachment complex
COL	type I collagen
CLSM	confocal laser scanning microscope
DMSO	dimethylsulfoxide
ECM	extracellular matrix
ER	endoplasmic reticulum
erk	extracellular signal regulated protein
F-actin	filamentous actin
FAK	focal adhesion kinase
FITC	Fluorescein Isothiocyanate
FN	

	fibronectin
GAP	GTPase activating protein
GEF	Guanine exchange factors
HBSS	Hank's balanced salt solution
LN	laminin-1
LPA	Lysophosphatidic acid
MAPKK	mitogen-activated protein kinase kinase also known as MEK
PI₃	phosphatidylinositol 3 kinase p-tyr, phosphotyrosine
TEM	transmission electron microscopy

Fig. 1.

Single optical sections through the basal region of epithelial tissues that were isolated –BL, then cultured in control media (**B**), Type I bovine collagen (COL, **D**), fibronectin (FN, **E**), laminin (LN, **F**) bombesin (**G**), or lysophosphatidic acid (**H**). The epithelia were fixed and stained with FITC-phalloidin then viewed on a Leica confocal laser scanning microscope. The F-actin microfilament bundle patterns appeared to have different characteristics following epithelia stimulation with different soluble molecules. The intensity of staining as measured by a standard scale. Black indicates no label and white indicates maximum signal (intensity wedge). All molecules stimulated the reorganization of the F-actin when compared to epithelia cultured without stimulating molecules (**A**,**B**). Single optical sections were obtained from the optical plane indicated by the dashed lines in the drawings of the corneal epithelial tissue. Individual basal cells were approximately 8–10 μm in diameter and each image was originally 50 μm^2 . Therefore, the schematic drawings are a larger scale than the micrographs. Phalloidin labels the F-actin associated with basal-lateral cell membranes, but not nuclei. Actin staining through the cytoplasmic blebs appears punctate in the en face optical section (**B**, arrowheads). Epithelium cultured in the presence of COL for 2 hr have extensive actin bundles that line up from cell to cell (**D**, arrowheads) across the field. Epithelium cultured with FN had shorter actin bundles (**E**, arrowheads), whereas an optical section from an epithelium cultured in the presence of LN (**F**, arrowheads) had longer actin bundles across the basal epithelium. The epithelium stimulated with bombesin (**G**) was optically sectioned at a tangential plane. The basal actin cytoskeleton (**G**, between the dashed lines) was reformed after 30 min exposure to bombesin. The upper right corner (**G**) was at the optical section through the basal cell nuclei and the individual cell size can be observed in the honeycomb pattern of the lateral cell membrane actin distribution. Lysophosphatidic acid induced reformation of the actin cortical mat within 30 min (**H**, arrowheads). Scale bars = 10 μm .

Fibronectin Binding to Embryonic Corneal Epithelia

Fig. 2.

Corneal epithelia were isolated -BL, incubated with media containing FITC-FN for short time intervals (5, 10, 15, or 30 min). The epithelia were rinsed, fixed, and viewed on the confocal microscope in the *xz* optical plane. The intensity of bound FITC-labeled FN was recorded at the basal cell surface (intensity wedge) in a nonuniform pattern. All settings (pinhole, laser power, voltage, and offset) were the same for all epithelia. Average relative intensity measurements were obtained for each epithelial group ($n = 3$). The FITC-FN increased from 0 to 30 min as analyzed with NIH Image (graph).

Fig. 3.

Corneal epithelia were isolated -BL, incubated with media containing FN or COL for a short time interval (0, 10, 15, 20, or 30 min). The cultures were rinsed and changed to control non-ECM media, then the tissues were incubated for a total time of 2 hr. The epithelia were fixed and stained with FITC phalloidin, then groups coded (numbered) and scored by at least two people as +, ±, or -ACM. The experiments were repeated at three times, with an n = 5-7 epithelia/group. Folded and damaged tissue was not scored. The epithelia reorganized the basal F-actin after 15 min exposures to FN or COL, however, 22% epithelia responded after 10 min. Greater than 80% of the epithelia responded to the ECM molecules following a 20-30 min exposure to the ECM molecules similar to epithelia incubated for 2 hr.

Fig. 4.

Several proteins were phosphorylated on tyrosine residues after epithelia were incubated in COL. Corneal epithelia ($n = 20\text{--}30/\text{group}$) were isolated $-BL$, incubated with media containing COL for various time intervals (**A**; 0–60 min). The epithelia were removed from culture and placed directly into SDS-PAGE sample buffer with 1 mM sodium orthovanadate and separated on either a 15% gel (**A**) or 7.5% gel (**B–D**). Tyrosine phosphorylated proteins were detected with antibodies to PY-20. Several proteins (mw 125, 60–70, and 40–45 kDa) appeared to increase or decrease phosphorylation with time. The phosphoproteins were identified by several methods: reprobing the same phosphotyrosine blot, separate parallel Western blots (**B**) and immunoprecipitations (**C,D**). Samples of the individual Western blots from 7.5% gels for

each protein (B) demonstrate that the >220 kDa protein was identified as tensin, the 190 kDa protein was p190 Rho-GAP, the 125–135 kDa protein was focal adhesion kinase (FAK) and the 60–70 kDa protein was paxillin. C: All phosphoproteins were immunoprecipitated from epithelia isolated –BL with PY20 following COL stimulation for 1 hr (+) compared to controls (–). The proteins were separated on 7.5% SDS-PAGE gels, and analyzed for tensin, FAK, paxillin, and p190Rho-GAP. Tensin, FAK, and paxillin co-immunoprecipitated by PY-20, however, p190Rho-GAP did not, indicating that the epitope was masked or the protein was not phosphorylated. D: Epithelia were isolated –BL, incubated with FN for 30 min, then immunoprecipitated with FAK, PAX, PY-20, or control (C). The immunoprecipitated proteins were separated on a 7.5% SDS-PAGE gel, then immobilized and incubated with antibodies specific for tensin (D). Tensin co-immunoprecipitated with FAK indicating that these two proteins are closely associated. Tensin did not co-immunoprecipitate with paxillin over control levels.

Fig. 5.

Western blot analysis of epithelial extracts cultured with or without COL (NT, 2 hr) to detect erk-2 (A), MEK-2 (B), MAP kinase activity (C), or PI₃ kinase (D). The 42 kDa protein, erk-2 was constant in control and COL-stimulated epithelia (A), but appeared to shift mobility slightly in the presence of COL. The same Western blot was reprobed for MEK-2 (B). The 42 kDa protein was erk-2 while the 44 kDa protein was MEK-2 (B). The protein levels of erk-2 and MEK-2 appeared constant in control and COL-stimulated epithelia (B). Activated erk-1 or erk-2 (denoted by *) were detected with an anti-human antibody that only binds to phosphorylated erk-1 and 2 (C). Phosphorylated erk-1 (44 kDa) and erk-2 (42 kDa) increased over time with COL-stimulation (C). The 85 kDa subunit of PI₃ kinase was expressed at low levels in blebbing epithelia isolated -BL (NT) but increased following 2 hr of COL-stimulation (D).

Fig. 6.

Herbimycin A blocked actin cortical mat reformation in a dose dependent manner (D). Corneal epithelia were isolated -BL, incubated with media containing LN, FN, or COL with a high (5 μ M) or medium (1.7 μ M) or low (0.17 μ M) concentration of Herbimycin A (HA) for 2 hr. The epithelia were fixed and stained with FITC phalloidin, then groups coded (numbered) and scored by at least two people as +, $\pm$, or -ACM. Examples of each type of score (+ACM [A], $\pm$ ACM [B], or -ACM [C]) indicate how the tissue was analyzed. The experiments were repeated at least three times with an $n = 5-7$ epithelia/group. Folded and damaged tissue was not scored. The ACM reformation stimulated by all substrates tested were inhibited by HA in a dose dependent manner (D). ACM reformation was most substantially blocked in epithelia

cultured in the presence of 5 μM HA(C), but the middle concentration (1.7 μM) also had a significant effect in this morphological assay (B,D).