Type I Collagen Structure Regulates Cell Morphology and EGF Signaling in Primary Rat Hepatocytes through cAMP-dependent Protein Kinase A

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Adhesion to type 1 collagen elicits different responses dependent on whether the collagen is in fibrillar (gel) or monomeric form (film). Hepatocytes adherent to collagen film spread and proliferate, whereas those adherent to collagen gel remain rounded and growth arrested. To explore the role of potential intracellular inhibitory signals responsible for collagen gel-mediated growth arrest, cAMP-dependent protein kinase A (PKA) was examined in hepatocytes adherent to collagen film or gel. PKA activity was higher in hepatocytes on collagen gel than on film during G1 of the hepatocyte cell cycle. Inhibition of PKA using H89 increased cell spreading on collagen gel in an EGF-dependent manner, whereas activation of PKA using 8-Br-cAMP decreased cell spreading on collagen film. PKA inhibition also restored ERK activation, cyclin D1 expression and G1-S progression on collagen gel, but had no effect on cells adherent to collagen film. Analysis of EGF receptor phosphorylation revealed that adhesion to collagen gel alters tyrosine phosphorylation of the EGF receptor, leading to reduced phosphorylation of tyrosine residue 845, which was increased by inhibition of PKA. These results demonstrate that fibrillar type 1 collagen can actively disrupt cell cycle progression by inhibiting specific signals from the EGF receptor through a PKA-dependent pathway.

INTRODUCTION

Adhesion to the extracellular matrix (ECM) is a key regulator of cell proliferation, differentiation, and apoptosis. Transmembrane adhesion receptors such as integrins link the ECM to the cytoskeleton, allowing both the biochemical and mechanical properties of the ECM to influence signal transduction cascades that regulate cell cycle progression (Assoian and Zhu, 1997; Aplin *et al.*, 1999; Giancotti and Ruoslahti, 1999; Schwartz and Assoian 2001). Although integrin-mediated adhesion is generally required for growth factor induced cell cycle progression, adhesion to certain ECMs can inhibit cell cycle progression. Growth inhibition by these ECMs likely plays a major role in maintaining tissue architecture and homeostasis, yet little is known about the mechanisms by which this occurs.

Adhesion to type I collagen can affect cell morphology, differentiation, and cell cycle progression, depending on its structure. When polymerized into fibrils, as it is mostly found *in vivo*, type I collagen inhibits growth of a number of cell types including smooth muscle cells (Koyama *et al.*, 1996), melanoma cells (Henriet *et al.*, 2000), glomerular epithelial cells (Schocklmann *et al.*, 2000), and hepatocytes (Hansen and Albrecht, 1999; De Smet *et al.*, 2001), whereas

adhesion to monomeric collagen stimulates cell cycle progression under similar culture conditions. Unlike loss of adhesion, however, which blocks cell cycle progression and also induces apoptosis, adhesion to collagen gels promotes survival and increased differentiated function (Ben-Ze'ev *et al.*, 1988; Gomez-Lechon *et al.*, 1998).

Potential mechanisms by which adhesion to fibrillar type 1 collagen inhibits cell cycle progression have been described. Decreased cyclin D1 expression (Hansen and Albrecht, 1999; Schocklmann *et al.*, 2000), and/or increased expression of cell cycle inhibitor proteins such as p21 or p27^{KIP} (Koyama *et al.*, 1996; Henriet *et al.*, 2000) have been demonstrated in various cell types in response to adhesion to fibrillar collagen. The mechanisms by which adhesion to fibrillar collagen modulates the signaling pathways that determine the expression levels of cell cycle regulatory proteins, however, are not well defined.

In primary rat hepatocyte cultures, cells adherent to polymerized collagen gels maintain a rounded morphology, express lower levels of cyclin D1, and do not enter S-phase despite the presence of mitogenic quantities of EGF (Hansen and Albrecht, 1999). More recent results suggest that inhibition of EGF signaling by adhesion to collagen gel occurs in part by inhibition of ERK activation, which is required for cell cycle progression on monomeric collagen (Fassett *et al.*, 2003). One signaling molecule that is both regulated by adhesion and is inhibitory of EGF signaling is cAMP-dependent protein kinase (PKA). PKA can limit the activity of raf (Cook and McCormick, 1993; Wu *et al.*, 1993), PAK (Howe and Juliano, 2000), and the EGF receptor (Barbier *et al.*, 1999), all of which are important to mitogenic signaling. Furthermore, agents that increase cAMP levels or activate PKA in hepatocytes also decrease cell cycle progression (Gjertsen *et al.*, 1995; Mellgren *et al.*, 1997). Activation of PKA can also decrease cell spreading and disrupt the actin cytoskeleton

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(Lamb *et al.*, 1988; Clegg *et al.*, 1989; Amieux *et al.*, 2002). Importantly, PKA is activated in fibroblasts upon detachment from the ECM, and inhibition of PKA restores growth factor activation of ERK in suspended cells (Howe and Juliano, 2000).

In this report, the potential role of PKA in growth arrest of hepatocytes by adhesion to polymerized type I collagen gel was investigated. It was demonstrated that PKA activity is differentially regulated by adhesion to polymerized or monomeric collagen and that PKA activity plays an important role in cell shape, cyclin D1 expression, and cell cycle progression in response to EGF. The growth inhibitory effect of PKA activation by polymerized collagen appears to occur in part at the level of EGF receptor phosphorylation, suggesting a novel mechanism in which adhesion to different forms of ECM may control cell growth and morphology by activating a PKA-dependent pathway that inhibits specific EGF-dependent cell cycle events.

MATERIALS AND METHODS

Reagents

Reagents: U0126 was purchased from Promega (Madison, WI). H89, myristoylated PKI, and 8-bromo cAMP were purchased from Calbiochem (La Jolla, CA). Antibodies used for Westerns include anti-rat cyclin E (cat. no. sc-481, Santa Cruz Biotechnology, Santa Cruz, CA) anti-phospho-tyrosine (cat. no. sc7020, Santa Cruz), anti-active-ERK, anti-ERK, anti-phosphoty-845 EGF receptor, and anti-phosphoty-1068 EGF receptor (cat. nos. 9102, 9101, 2231, and 2234, respectively, Cell Signaling, Beverly, MA), anti-cyclin D1 (cat. no. 06-137, Upstate Biotechnology; Lake Placid, NY), anti-EGF receptor (cat. no. 06-847, Upstate Biotechnology), and anti-G3PDH (cat. no. 8245 100, AbCam, Cambridge, United Kingdom).

Hepatocyte Culture

Primary rat hepatocytes were obtained by collagenase perfusion of adult Lewis rat liver (Seglen, 1976), followed by purification through a Percoll gradient (Sigma Chemical Company, St. Louis, MO). Only cells from harvests yielding >90% viability were used. Hepatocytes were plated at subconfluent density (10–12,000 cells/cm²) in serum-free William's medium E (Invitrogen, Grand Island, NY) with the following additives as previously described (Hansen and Albrecht, 1999): epidermal growth factor (EGF; 10 ng/ml, Collaborative Research, Bedford, MA), insulin (20 mU/ml, Sigma), dexamethasone (5 nM, Sigma), sodium pyruvate (1 mM, Invitrogen), ascorbic acid (50 µg/ml, Invitrogen), and penicillin/streptomycin (100 U/ml, Irvine Scientific, Santa Ana, CA). Cultures were refed daily. Cultures were always performed in the presence of growth factors unless otherwise stated. "Time 0" samples represent freshly isolated cells in suspension at 4°C with minimal medium (no growth factors) before plating.

Collagen Substrate Preparation

Type I collagen (Vitrogen 100, Collagen Biomaterials, Palo Alto, CA) was coated onto nonadhesive Petri dishes to produce either a monomeric coat of collagen referred to as collagen film or a polymerized form referred to as collagen gel. Collagen film was prepared by coating dishes with collagen diluted in a basic, carbonate buffer (pH 9.4) as previously described (Hansen and Albrecht, 1999). To produce a collagen gel substrate, 50 µl NaOH was added to each ml of 5× Williams Media E. One part 5× Williams Media E (+NaOH), were mixed with four parts Vitrogen 100 (final concentration ~2.4 mg/ml), added to a Petri dish at ~1 ml/10 cm dish and incubated at 37°C for 1 h. After coating, all plates were washed twice in phosphate-buffered saline (PBS) and incubated at least 20 min in 1% bovine serum albumin (BSA) in Williams E medium to block any noncoated sites. Hepatocyte attachment is equivalent on both substrates. Collagen gel overlays were accomplished by mixing five parts complete Williams E and one part Vitrogen 100 (final collagen concentration ~0.5 mg/ml). Media were removed from cells and replaced with overlay. Fresh media were added on top of the overlay after polymerization.

DNA Synthesis

DNA synthesis is measured by adding [³H]thymidine at 1.2 µCi/well (specific activity = 50 Ci/mmol, ICN Biomedicals, Costa Mesa, CA) to 96-well plate cultures at the indicated hours after plating. [³H]thymidine incorporation into newly synthesized DNA was determined by harvesting cell lysates onto filter paper using a cell harvester (Brandel, Gaithersburg, MD) and quantitation by scintillation counting.

Protein Isolation, Immunoprecipitation, and Western Blot

Cultured hepatocytes were removed from the plates by washing once with ice-cold PBS and then scraping in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, leupeptin, and pepstatin, 1 mM sodium vanadate, 1 mM NaF). Immunoprecipitation was carried out as previously described (Albrecht and Hansen, 1999). Western blot analysis was performed using ~30 µg of cellular protein per lane as previously described (Albrecht and Hansen, 1999).

PKA Assay

Assay for cAMP-dependent protein kinase A was performed as previously described (Day *et al.*, 1989). Briefly, cells were lysed, sonicated, and centrifuged, and total protein was determined. Equal protein was added to reaction mixture along with substrate (Kemptide, a synthetic peptide containing the consensus PKA phosphorylation sequence), and 5 µCi [³²P]ATP (3000 Ci/mmol, Amersham, Piscataway, NJ). After 30°C incubation for 10 min, 25 µl of reaction mixture was spotted onto Whatman P81 paper, washed extensively in 75 mM phosphoric acid, dried, and quantitated by scintillation. The PKA assay was also performed in the presence of PKI, a peptide inhibitor of PKA. These values were similar to background counts obtained in the absence of Kemptide substrate, indicating the specificity of the assay (our unpublished data).

Cell Size Measurements

Hepatocytes were cultured for the indicated period of time, fixed with 4% paraformaldehyde/phosphate-buffered saline pH 7.4, washed three times in PBS, and then stained for 5 min with Coomassie blue/methanol solution. Cells were then washed five times in PBS and photographed measured using computerized image analysis with Adobe Photoshop 6.0 (San Jose, CA). At least 50 randomly selected cells were analyzed and averaged for each condition.

Scanning Electron Microscopy

Hepatocytes were cultured on thermamax plastic coverslips (Nunc, Rochester, NY) that were coated with collagen film or collagen gel as described above. At the time of analysis, coverslips were washed three times in Williams E, fixed in 1% glutaraldehyde for 2 h at 4°C, washed three times for 5 min with 0.1 M Na cacodylate, treated with osmium tetroxide for 1 h at room temperature, washed three times with diH₂O, incubated in 50% ethanol for 10 min, washed in 70% ethanol for 10 min, and then stored until use. Scanning Electron Microscopy (SEM) was performed using a Hitachi S-4700 FEG scanning electron microscope (Hitachi, Pleasanton, CA).

RESULTS

Interaction with Polymerized Collagen Increases PKA Activity

Previously it was demonstrated that EGF-dependent activation of the p42/44 ERK pathway and G1-S progression is disrupted in hepatocytes adherent to polymerized (fibrillar) type I collagen gel, but not on monomeric type I collagen film (Fassett *et al.*, 2003). To determine if polymerized collagen gel had a dominant inhibitory effect on cell cycle progression or if growth arrest was simply due to lack of sufficient positive ECM-generated signals, experiments were designed in which hepatocytes were first cultured on collagen film, which stimulates cell cycle progression, followed 12 h later with an overlay of type I collagen gel. This "collagen sandwich" configuration has been described in the literature (Dunn *et al.*, 1989) and promotes a highly differentiated phenotype. After addition of the gel overlay, the hepatocytes stopped spreading, but still remained more spread than the hepatocytes on collagen gel alone (Figure 1A). Using computerized image analysis, the average spread area of overlaid cells was determined to be 41% (±6%) the area of film, whereas gel was 31% (±3%) the size of cells on film. DNA synthesis assay performed 54–74 h after initial plating (42–62 h after collagen gel overlay) demonstrated a significant inhibition of DNA synthesis in the presence of the gel overlay compared with cells plated on film over the same time period without overlay (Figure 1B, *p* < 0.01). These data indicate that collagen gel actively inhibits cell cycle

Figure 1. Collagen gel actively inhibits DNA synthesis. (A) Hepatocytes were plated on collagen film or gel in the presence of growth factors. Twelve hours later, a solution of type I collagen in William's E medium was overlaid onto cells in a subset of the film plates, which formed a layer of polymerized collagen on top of the cells. Photos were taken of cultures at 48 h. (B) [^3H]thymidine incorporation was assessed as a measurement of DNA synthesis 54–74 h after initial plating. Data presented are mean $\pm$ SD of quadruplicate samples from one representative experiment. (** $p < 0.01$).

progression even in cells already progressing through G1 phase, suggesting that fibrillar collagen interaction with hepatocytes induces a specific cell cycle inhibitor.

One signaling molecule that blocks mitogenic signaling upon activation in some cell types is the cAMP-dependent protein kinase (PKA). To investigate the possibility that PKA may play a role in different EGF signaling on distinct forms of collagen, the activity of PKA was measured at different times in hepatocytes adherent to monomeric collagen film, or polymerized collagen gel. As shown in Figure 2, hepatocytes adherent to polymerized collagen have higher levels of PKA activity than those adherent to monomeric collagen and this higher activity lasts throughout the first 48 h of culture (i.e., throughout G1 phase of the hepatocyte cell cycle; $p < 0.05$ for each time point tested). In addition, collagen overlay, when applied to hepatocytes on film 12 h after plating, led to a significant increase in PKA activity at 24 h compared with cells cultured for the same time period on film alone ($p < 0.05$). The level of PKA activity induced by collagen gel overlay was similar to that measured in cells on collagen gel for the entire 24 h culture.

PKA Regulates Morphological Response to EGF

Because PKA levels appeared higher in the rounded cells on polymerized collagen than in spread cells on monomeric collagen, the effects of PKA activity on hepatocyte morphology were investigated by blocking PKA activity using the PKA specific inhibitor H89. Using an antibody specific for phosphorylated PKA substrates, a substantial reduction in phosphorylation in many cellular proteins is observed in the presence of H89, indicating its function in reducing PKA activity (our unpublished data). Surprisingly, treatment of hepatocytes with H89 at the time of plating dramatically increased cell spreading (Figure 3, A–C). Conversely, treat-

Figure 2. PKA activity is increased on polymerized collagen. (A) PKA activity was measured in hepatocytes adherent to collagen film or collagen gel at the indicated time points. T = 0 (black bar) indicates freshly isolated cells before plating. PKA activity was determined by measuring incorporation of radiolabeled phosphate into a PKA substrate peptide (Kemptide) added to cell lysates and subtracting background counts obtained in the absence of substrate. (B) PKA assay was performed as above, with the addition of the overlay condition, in which hepatocytes were plated onto collagen film for 12 h, followed by collagen gel overlay. PKA activity was assessed 24 h after initial plating for all conditions (12 h after overlay addition). Error bars represent SD from duplicate samples of representative experiments (from 6 PKA assay experiments and 3 overlay experiments; * $p < 0.05$; NS, not significant).

ment of cells adherent to collagen film with PKA agonist 8Br-cAMP had an effect similar to that of adhesion to collagen gel, causing cells to remain rounded and less spread (Figure 3, A and C). At 48 h the difference between collagen film and gel was still evident. If only solitary cells were measured, the difference in size was even more pronounced (Figure 3C, inset at 48 h), suggesting cell/cell adhesion can increase spreading across a basal layer of collagen gel. The ability to spread on collagen gel under these conditions in response to PKA inhibition was dependent on EGF, because H89 treated cells stimulated with EGF spread substantially by 48 h, whereas H89-treated cells in the absence of EGF did not spread (Figure 3, B and C). It is also important to note that although H89-induced spreading on collagen gel was decreased by ~70% in the absence of EGF, spreading on collagen film was only diminished by ~30% in the absence of EGF (our unpublished data), suggesting that although EGF is important for spreading on collagen gel, adhesion to collagen film can induce substantial spreading that is independent of EGF. Thus, although malleable gels such as collagen gels were traditionally thought to inhibit spreading because of the inability to resist cytoskeletal tension at adhesion sites, these results suggest that inhibition of cell spreading on type I polymerized collagen is due in part to induction of elevated PKA activity that inhibits EGF-induced spreading.

Figure 3. Inhibition of PKA increases cell spreading on polymerized collagen. (A) Hepatocytes were plated on collagen film with or without the PKA agonist, 8Br-cAMP (1 mM) and on collagen gel with or without the PKA inhibitor H89 and then at 24 h, cells were either photographed using phase contrast microscopy with Varel optics or processed for scanning electron microscopy (SEM). (B) Hepatocytes were photographed at 48 h after culture on film, on collagen gel, on collagen gel in the presence of 3 μM H89, or on collagen gel with 3 μM H89, but in the absence of EGF. (C) Cell areas at 48 h for the above conditions were analyzed by computer image analysis of at least 50 randomly selected cells per condition. Inset, cell area at 48 h when only solitary cells were measured.

Inhibition of PKA on Collagen Gel Restores DNA Synthesis

It has long been recognized that the ability of the ECM to support cell spreading correlates with its ability to promote cell cycle progression (Folkman and Moscona, 1978; Ingber *et al.*, 1987; Mooney *et al.*, 1992; Hansen *et al.*, 1994). To determine the effects of PKA inhibition on cell cycle progression, DNA synthesis was measured in hepatocytes treated

with varying concentrations of H89. Inhibition of PKA activity on collagen gel with moderate doses of H89 (1.5–6 μM) significantly increased DNA synthesis on collagen gel compared with dimethyl sulfoxide (DMSO) controls on gel ($p < 0.005$), and DNA synthesis levels were similar to that seen on film DMSO controls. However, inhibition of PKA on collagen film had no significant effect on G1-S progression until higher doses (6–12 μM) were used, and DNA synthesis

Figure 4. Inhibition of PKA activity increases DNA synthesis on collagen gel. (A) Hepatocytes were cultured on collagen film or collagen gel in Williams's E medium containing EGF in the presence of increased amounts of H-89, and DNA synthesis was measured by incorporation of [3 H]thymidine between 52 and 72 h. NS, not significant; ** $p < 0.05$, compared with matrix/DMSO, -) (B) Hepatocytes adherent to collagen gel were cultured with increasing amounts of myristoylated PKI for 0–24 h after plating and DNA synthesis was measured by thymidine incorporation between 52 and 72 h. (NS, not significant; ** $p < 0.01$) (C) Hepatocytes adherent to collagen film or collagen gel were exposed to 3 μ M H89 for either 24 or 48 h in the presence or absence of EGF. [3 H]thymidine incorporation was measured between 48 and 72 h. NS, not significant; * $p = 0.047$; ** $p < 0.01$; ND, not determined.

actually decreased (Figure 4A). The increase in DNA synthesis was not likely due to H89's "nonspecific" inhibition of other molecules, such as rho kinase (Leemhuis *et al.*, 2002), because treatment of cells on gel with rho kinase inhibitor Y2697 was unable to induce DNA synthesis of hepatocytes on collagen gel (our unpublished data). To further test the specificity of PKA inhibition, a different inhibitor of PKA activity was utilized. A myristoylated form of PKI, a PKA-inhibitory peptide that blocks the substrate-binding site of PKA, was added to hepatocyte cultures on collagen gel for the first 24 h of culture. This treatment also significantly increased hepatocyte DNA synthesis on collagen gel compared with controls ($p < 0.01$ at 4 μ g/ml; Figure 4B), again to a level similar to that on film control ($p > 0.05$). Because inhibition of PKA is able to restore EGF-dependent G1-S progression on collagen gel, but has little effect on collagen film, this is further evidence that PKA-mediated inhibition of hepatocyte cell cycle progression is a response specific for polymerized and not monomeric collagen.

Hepatocytes in this system typically reach the growth factor restriction point around 44–48 h on collagen film (Albrecht and Hansen, 1999). Removal of H89 at 24 h after plating only slightly diminished DNA synthesis compared with H89 removal at 48 h, which corresponds to entry into S phase (Figure 4C; not significant for film, $p = 0.047$ for gel comparing 0–48 to 0–24 h), suggesting the inhibitory effects of PKA activity on cell cycle progression takes place during early and mid-G1 of the cell cycle (0–24 h). H89 also was not able to induce DNA synthesis in the absence of EGF, suggesting that H89 was restoring EGF signaling rather than an EGF-independent pathway to the cell cycle (Figure 4C; $p < 0.01$ comparing +EGF to -EGF for each condition).

Inhibition of PKA Reduces Collagen Gel-mediated Inhibition of p44/42 ERK and Cyclin D1 Expression

PKA is known to antagonize EGF signaling pathways and mitogenesis (Cook and McCormick, 1993; Wu *et al.*, 1993).

Previously we have shown that adhesion to polymerized collagen inhibited EGF-dependent G1-S progression, and this inhibition was associated with lower levels of cyclin D1 expression (Hansen and Albrecht, 1999), due in part to decreased ERK activity in late G1 (around 36–48 h after plating; Fassett *et al.*, 2003). Overexpression of cyclin D1 (Hansen and Albrecht, 1999) or expression of activated mutants of the Ras signaling pathway were able to restore DNA synthesis on collagen gel (Fassett *et al.*, 2003). In smooth muscle cells, adhesion to fibrillar collagen gel does not effect cyclin D1 levels, but there is an increase in the levels of a cell cycle inhibitor protein p27^{KIP} (Koyama *et al.*, 1996). To determine if PKA plays a role in collagen gel-mediated inhibition of p42/44 ERK activity or regulates cyclin D1 or p27^{KIP} expression, PKA was inhibited and these cell cycle regulators were analyzed by Western blot. Both p42/44 ERK activation and cyclin D1 expression were increased on collagen gel after inhibition of PKA (Figure 5A). Blocking PKA for only the first 24 h was sufficient for inducing an increase in ERK activity during late G1 and S. This level of ERK activation or cyclin D1 expression did not equal that found on collagen film however, suggesting that other signaling pathways that are not restored on collagen gel by PKA inhibition alone. p27^{KIP} levels, lower on collagen gel than on collagen film at these time points, were increased by inhibition of PKA. Cyclin E levels or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) levels, which are not significantly affected by adhesion to gel, also did not appear to be greatly affected by PKA inhibition. Treatment of hepatocytes with myristoylated PKI also was able to increase cyclin D1 expression on collagen gel, and had little effect on cyclin E (Figure 5B). These results suggest that adhesion to collagen gel specifically blocks cyclin D1 through a PKA-dependent mechanism. Further analysis of the interaction between the upstream EGF signaling, collagen structure and PKA on cell cycle progression demonstrated that not all of the EGF-dependent signaling

Figure 5. Inhibition of PKA activity restores EGF signaling to ERK and cyclin D1. (A) Hepatocytes were cultured in the presence or absence of 3 μ M H89 for the first 24 h of plating. Cell lysates were collected at 52 and 72 h after plating, and expression of the indicated proteins was analyzed by Western blot. (B) Hepatocytes were cultured on collagen gel and incubated with myristoylated PKI (1 μ g/ml) for the first 24 h of culture. Cell lysates were collected at 60 h and analyzed by Western blot. (C) Hepatocytes were cultured on film with or without EGF, or on gel with EGF in the presence or absence of 3 μ M H89 from 0 to 48 h, and lysates were collected at the indicated times for Western blot analysis.

pathways are disrupted by adhesion to collagen gel. For instance, EGF stimulation increases the levels of cyclin E and cdc2 expression above levels found in untreated cells (Figure 5C). Both of these EGF-dependent events appeared unhindered by adhesion to polymerized collagen. However, EGF-dependent induction of cyclin A, another late G1/S phase cell cycle promoting gene, was blocked by adhesion to collagen gel, but was partially restored by H89 treatment, as was cyclin D1. Cdc2 tyrosine-15 phosphorylation and dephosphorylation, which are involved in the timing of mitosis (Krek and Nigg, 1991; Borgne and Meijer, 1996) also appeared to be inhibited by adhesion to collagen gel and were restored by H89 treatment (Figure 5C). Thus, although not all EGF-dependent events are blocked by adhesion to polymerized collagen, a number of events critical to G1-S progression as well as progression out of S phase appear to be blocked by adhesion to collagen gel in a PKA-dependent manner. This suggests that EGF is able to bind and activate the receptor to some degree, but some aspect of EGF signaling at the level of activation or downstream is disrupted.

Cyclin D1 may be induced by other signaling molecules, such as the small GTPase rac, independent of ERK (Welsh *et al.*, 2001). To determine if the increase in cyclin D1 in response to PKA inhibition was a result of restoring EGF signaling to ERK, cells were treated with H89 on collagen film or collagen gel for 48 h and then treated with U0126, an inhibitor of MEK, the upstream activator of ERK, for the last 24 h of culture (48–72 h). Protein lysates obtained at 72 h and

Figure 6. Increase in cyclin D1 by PKA inhibition is dependent on MEK activity. (A) Hepatocytes were cultured for 48 h with 3 μ M H89 or equivalent concentration of DMSO and then treated between 48 and 72 h with DMSO or 25 μ M U0126. Cell lysates were collected at 72 h and analyzed for cyclin D1 expression or G3PDH by Western analysis. (B) Hepatocytes were treated as in A and DNA synthesis was measured between 48 and 72 h.

DNA synthesis between 48 and 72 h was then measured. Results demonstrate that inhibition of MEK blocks cyclin D1 expression on collagen film as well as H89-mediated induction of cyclin D1 on collagen gel (Figure 6A). Inhibition of MEK also reduced the DNA synthesis induced by PKA inhibition on collagen gel (Figure 6B; $p < 0.01$) as well as that normally seen on collagen film as previously described (Figure 6B; $p < 0.01$; Fassett *et al.*, 2003). These data demonstrate that PKA blocks EGF signaling to ERK and subsequent cyclin D1 expression and G1-S progression on collagen gel. Removal of EGF at 48 h has no effect on DNA synthesis in the presence of H89 (our unpublished data), suggesting that progression into and possibly through S phase is dependent on MEK during this 48–72-h period, but no longer dependent on EGF. EGF was required to drive the cell cycle to this point, and adhesion to collagen gel specifically blocks this progression through activation of PKA.

Tyrosine Phosphorylation of EGF Receptor Is Affected by Adhesion to Collagen Film or Gel

There is evidence that integrin-mediated adhesion can activate the EGF receptor (EGFr) even in the absence of EGF (Moro *et al.*, 1998; Moro *et al.*, 2002). It has also been demonstrated that PKA can directly transmodulate the EGFr (Barbier *et al.*, 1999). Previous results from this laboratory suggest that inhibition of EGF signaling to ERK on polymerized collagen gel occurs upstream or at the level of ras activation (Fassett *et al.*, 2003). Thus it was of interest to determine if adhesion to polymerized collagen was affecting EGF signaling at the level of EGFr expression or activation. The results in Figure 5 suggested some level of EGFr activity was persisting on collagen gel based on cyclin E expression, yet its activity required for cyclin D1 expression and activation of ERK appeared disrupted. To examine the ability of EGF to activate the receptor, immunoprecipitation (IP) followed by Western blot analysis was performed. These results demonstrated no detectable difference in the amount of

Figure 7. Polymerized collagen inhibits EGF receptor phosphorylation. (A) To analyze EGF receptor phosphorylation on collagen film or gel and in response to PKA modulation, hepatocytes were cultured up to 4 h on collagen film or gel, followed by immunoprecipitation with anti-phosphotyrosine antibody and Western blot analysis with anti-EGFR antibody. (B) A more extensive time course of EGFR phosphorylation was examined from 2 to 24 h on collagen film (F) or gel (G) in culture, analyzed by anti-phosphotyrosine Western blot using antibodies for total phospho-tyrosine and total EGF receptor. (C) Densitometry of bands from at least three different blots as in B was performed and compared relative to the maximal value (* $p < 0.05$ comparing film and gel).

total phosphotyrosine-containing EGFR on gel or film over the first 4 h in culture (Figure 7A), suggesting that the EGFR signaling pathway was not compromised upon initial plating onto collagen gel. However, examination of a more extensive time course revealed that although overall phosphorylation of the EGFR is decreased on both film and gel at later times, a slightly higher level of EGFR phosphorylation is maintained for at least 24 h on film (Figure 7, B and C, first panel; $p < 0.05$ at 6–9 and 24 h, comparing gel to film).

Because the EGFR contains multiple tyrosine residues with different signaling functions, further analysis of specific tyrosine residues of the EGFR was also undertaken. Tyrosine 845 phosphorylation of the EGFR is mediated by Src in response to adhesion (Biscardi *et al.*, 1999; Moro *et al.*, 2002) and is also important for EGF-mediated DNA synthesis as well as DNA synthesis induced by transactivating receptors that signal through the EGFR (Biscardi *et al.*, 1999; Tice *et al.*, 1999). Tyrosine 1068 is also phosphorylated in response to adhesion via integrins (Moro *et al.*, 2002) and is an autophosphorylation site for the EGFR (Downward *et al.*, 1984, 1985). When cells were plated on collagen gel, phosphorylation of Tyr-845 never attained the same level of phosphorylation and decreased more rapidly in comparison to cells plated on film, ($p < 0.05$ at all time points when comparing gel relative to film at same time point), whereas tyrosine 1068 phosphor-

ylation appeared similar on both substrates (Figure 8, A and C). It is also evident that by 24 h of culture, the overall levels of the EGFR are much reduced, as previously demonstrated in response to continuous stimulation (Gross *et al.*, 1983; Helin and Beguinot, 1991). Treatment of hepatocytes with H89 was able to increase both total EGFR phosphorylation ($p < 0.05$ at 0.5 and 2.5 h) and Tyr-845 phosphorylation on collagen gel ($p < 0.05$ for 2.5 h; not significant at 0.5 h), but had little effect on Tyr-1068 ($p > 0.05$), suggesting that PKA plays a specific role in the diminished phosphorylation of Tyr-845 in hepatocytes adherent to collagen gels (Figure 8C; no quantitation provided). As this site has been demonstrated to be regulated by Src through an integrin-dependent mechanism (Moro *et al.*, 2002), it suggests that PKA may play a role in uncoupling the cooperative relationship between the EGFR and integrin signaling complexes in signaling through G1 toward cell cycle progression.

The differences in overall tyrosine phosphorylation of the EGFR as well as tyr 845 between film and gel appear to be greatest later in the culture period (between 9 and 24 h). However, at the same time (~24 h), overall phosphorylation as well as expression of the EGFR is down-regulated (Figure 7B), and thus the role for EGFR phosphorylation in the late G1 ERK activity and cyclin D1 expression typically seen around 48 h is not clear. To determine if the EGFR plays a role in late signaling to ERK and cyclinD1, EGF was removed after 12 h of culture and the cells were incubated for an additional 36 h in the presence or absence of H89 without EGF. H89 treatment from 0 to 48 h of hepatocytes on collagen film did not alter ERK phosphorylation or cyclin D1 expression (our unpublished data). Figure 9A demonstrates that removal of EGF by 12 h reduces the late G1 activation of ERK and cyclin D1 expression on collagen film (compare to control exposed to continuous EGF), suggesting a requirement for EGF beyond 12 h of culture in order to obtain high levels of cyclin D1 expression. Reintroducing EGF at 48 h on film strongly induces ERK phosphorylation, which is followed by increased cyclin D1 expression 12 h later. On collagen gel, EGF reintroduced at this later stage did not induce strong ERK phosphorylation or cyclin D1 expression, but ERK phosphorylation and cyclin D1 expression was restored in cells on gel treated with H89 from 0 to 48 h (Figure 9A). This suggests that EGF signaling during these later times on film and gel are important for driving ERK activation and cyclin D1 expression, and elevated PKA activity reduces this late G1 EGFR signaling on collagen gel. Addition of EGF at 48 h in the absence of prior EGF stimulation did not result in cyclin D1 expression 12 h later (our unpublished data), suggesting that both early and late phases of EGF stimulation are important for inducing cyclin D1 expression.

Examination of EGFR phosphorylation during this late phase of signaling (Figure 9B) demonstrates that after EGF readoption, Tyr-845 and Tyr-1068 phosphorylation of the EGFR is inhibited on collagen gel relative to film ($p < 0.05$ for Tyr-845, $p < 0.01$ for Tyr-1068, no quantitation provided), but partially restored by inhibition of PKA ($p < 0.05$ for Tyr-1068, $p < 0.001$ for Tyr-845, gel vs. gel + H-89). It is also evident that reexpression of the EGFR after EGF reintroduction is stronger on collagen film than on collagen gel, and this reexpression is slightly increased on gel in response to H89. Similar to the initial response to EGF, however, Tyr-1068 did not appear to be as strongly affected by H89 treatment, suggesting that inhibition of Tyr-845 phosphorylation is a specific target of adhesion signaling by collagen gel in a PKA-dependent manner. Because the phosphorylation of Tyr-845 is Src-dependent, its phosphorylation on gel +/-

Figure 8. Polymerized collagen inhibits phosphorylation of specific tyr residue in EGFR in a PKA-dependent manner. (A) EGFR phosphorylation on specific tyr residues (Tyr-845 and Tyr-1068) was examined from 15 min through 24 h on collagen film (F) or gel (G) in culture. (B) Densitometry of bands from at least three different blots as in A was performed and compared relative to the maximal value. (* $p < 0.05$ comparing film and gel) (C) Hepatocytes were treated with H89 or DMSO for 30 min before addition of EGF for 30 min or 2.5 h. Lysates were analyzed by Western blot for p-tyrosine, tyrosine residue 845, tyrosine residue 1068, and total EGF receptor.

H89 was examined in the presence or absence of the Src inhibitor, PP2. The restoration of Tyr-845 phosphorylation, as well as ERK induction, by EGF readdition is inhibited by PP2 (Figure 9C), confirming the involvement of Src in Tyr-845 phosphorylation and downstream ERK activation.

DISCUSSION

Studies presented here reveal a novel ECM regulatory mechanism in which adhesion to fibrillar, but not monomeric, type I collagen actively blocks EGF signaling to ERK, cyclin D1 and DNA synthesis through a PKA-dependent mechanism. This mechanism helps to explain the consistent observation that, although adhesion to ECM is generally required for nontransformed cells to progress through the cell cycle in response to growth factors, not all ECMs support proliferation of all cell types. The differences in the abilities of certain ECMs to regulate proliferation or differentiation within the context of an entire organ or organism are likely to play an important role in maintaining tissue homeostasis in vivo. Defining specific molecular mechanism(s) of ECM-dependent regulation of growth and differentiation thus provide valuable insight into these processes.

Previous studies from this laboratory demonstrated that initial ERK activation after cell plating is similar on collagen film and gel, but a second sustained peak in ERK activity that occurs in late G1 phase on collagen film is absent on collagen gel (Fassett *et al.*, 2003). This second sustained activation of ERK is required for progression into S phase, whereas the initial peak of ERK activity is not required for cell cycle progression on a permissive matrix. Other studies demonstrate that sustained ERK activity is required for cyclin D1 expression and G1-S transition (Weber *et al.*, 1997; Roovers *et al.*, 1999; Bottazzi *et al.*, 2001) and is dependent on adhesion and an intact cytoskeleton (Roovers *et al.*, 1999; Bottazzi *et al.*, 2001). Results presented here suggest that inhibition of PKA during early and mid-G1 was able to

restore cell morphology and perhaps a cytoskeletal architecture that more efficiently transduced the late G1 MEK-dependent signals. Furthermore, the removal of EGF at 12 h and subsequent loss of ERK activation and cyclin D1 expression suggests that at least two phases of EGF stimulation in early and late G1 are required to drive cyclin D1 expression. PKA inhibition by H89 during early and mid-G1, corresponding to the time during which PKA activity is higher on collagen gel, appears to prevent collagen gel-dependent inhibition of late ERK activation. The increased activation of ERK and cyclin D1 expression around 48 h in cells with H89 for either 24 or 48 h suggests that fibrillar collagen inhibits cell cycle progression in hepatocytes by interfering with cell cycle competence, as defined in the competence/progression model of cell cycle control (Stiles *et al.*, 1979).

PKA has previously been shown to inhibit EGF signaling at a number of points by down-regulating the activity of the EGF receptor (Barbier *et al.*, 1999), inhibiting the activation of raf (Cook and McCormick, 1993; Wu *et al.*, 1993; Hu *et al.*, 1999), or inhibiting p21 activated kinase (PAK; Howe and Juliano, 2000). Loss of adhesion activates PKA, which blocks growth factor signaling to ERK by inhibiting PAK (Howe and Juliano, 2000). PKA has also been shown to disrupt the actin cytoskeleton and decrease cell spreading (Lamb *et al.*, 1988; Clegg *et al.*, 1989; Amieux *et al.*, 2002). An intact cytoskeleton is a critical component of growth factor- and adhesion-dependent signaling to the G1-S transition (Assoian and Zhu, 1997; Bottazzi *et al.*, 2001; Schwartz and Assoian, 2001). When hepatocytes on collagen gel were treated with H89 to block PKA activity, EGF signaling to ERK and cyclin D1 was restored. Cell spreading was also associated with the increase in ERK activation and cyclin D1 expression in response to PKA inhibition, suggesting that PKA activity induced by adhesion to collagen gel may be involved in inhibiting morphological or cytoskeletal structures that promote ERK activation and cyclin D1 expression.

Figure 9. EGFr phosphorylation and subsequent cell cycle events are reduced in hepatocytes on gel in response to additional EGF at later time points. (A) Hepatocytes were cultured for 12 h with EGF on film or gel in the presence or absence of H89. After 12 h of treatment, the media was removed and replaced with media ($\pm$ H89) free of EGF for 36 h. New media were added with or without EGF at 48 h, and lysates were collected at the indicated time points and analyzed for phospho-ERK, cyclin D1, and GAPDH by Western blot. Controls exposed to EGF for 48 h are also shown (48). (B) Tyrosine residues 1068 and 845 and total EGF receptor levels were analyzed in lysates collected after 48 h of continuous EGF treatment (control, C) or 36 h after EGF withdrawal with or without readdition of EGF. H89 was present for only the first 48 h. (C) The role of Src in EGFr Tyr-845 phosphorylation on film or gel $\pm$ PKA inhibitor was examined by culturing hepatocytes as in B in the presence or absence of Src inhibitor, PP2.

H89 had no effect on cells adherent to film, demonstrating that PKA inhibitory activity is specific to collagen gel.

Adhesion even in the absence of EGF can signal through the EGF receptor (Moro *et al.*, 1998, 2002). PKA has been shown to directly phosphorylate and inactivate the EGF receptor (Barbier *et al.*, 1999). The finding that adhesion to collagen gel increases PKA activity and decreases EGF receptor phosphorylation suggests that adhesion to fibrillar collagen acts in the opposite manner of growth-permissive adhesion, that is, to directly suppress adhesion-dependent EGF signaling. Thus one difference between a growth supportive ECM and one that inhibits growth may be its effect on PKA activity and subsequent effects on growth factor signaling and cell shape.

The increase in cell spreading, ERK activation, cyclins D1 and A expression, and DNA synthesis by PKA inhibition

requires EGF, suggesting that the EGF-dependent signaling required for cell spreading and G1 progression is being blocked by PKA. However, not all EGF-dependent signaling was blocked by adhesion to collagen gel. Cyclin E and cdc2 expression were both increased similarly on collagen gel and film. Consistent with the findings that only some EGF-dependent signals were disrupted by adhesion to gel is the different pattern of phosphorylation of particular EGF receptor tyrosine residues in cells adherent to collagen film and gel. Tyr 1068 is an autophosphorylation site on the EGF receptor that is activated by integrin adhesion as well as EGF stimulation (Moro *et al.*, 2002). This site was activated by EGF similarly on collagen film or collagen gel. In contrast, Tyr 845, which is also activated by adhesion and EGF stimulation (Moro *et al.*, 2002), showed reduced phosphorylation in cells attached to collagen gel. Tyr 845 phosphorylation of

the EGF receptor is carried out by Src (Sato *et al.*, 1995), which associates with the EGF receptor in response to adhesion (Moro *et al.*, 2002). It has also been demonstrated that Tyr 845 is important for EGF-induced DNA synthesis (Biscardi *et al.*, 1999) as well as zinc-induced Ras activation (Wu *et al.*, 2002), and that Src and the EGF receptor cooperate to induce cellular transformation (Tice *et al.*, 1999). An increase in Tyr 845 phosphorylation in response to H89 suggests that PKA activity on collagen gel may disrupt interaction between the EGF receptor and Src, but have less effect on EGF-induced autophosphorylation. It is also possible that the increased ability of cells to spread on collagen gel in response to H89 may relate to increased integrin activation and subsequent integrin/EGF receptor interactions, suggesting an indirect effect of PKA on the EGF receptor.

The mechanism by which either adhesion to type I collagen fibrils or loss of adhesion activates PKA is not known, nor is it clear if PKA activation occurs through a similar mechanism in each situation. In general, ECM-mediated cell cycle inhibition can result from biochemical and/or physical properties of the ECM/cell contact. For example, adhesion to laminin through $\alpha_2\beta_1$ integrins fails to seed the formation of cytosolic signaling complexes and cytoskeletal organization important for growth factor signaling in endothelial cells, whereas $\alpha_5\beta_1$ -mediated adhesion to fibronectin does promote proliferative signaling in the same cells (Mettouchi *et al.*, 2001). Different integrins, as well as nonintegrin receptors such as DDRs (Shrivastava *et al.*, 1997; Vogel *et al.*, 1997), bind to distinct regions within type I collagen, depending on whether the collagen is in native or denatured conformation (Gullberg *et al.*, 1990, 1992). Thus these or other specific receptors may play a role in differential signaling by collagen conformation.

Mechanical or spatial properties of the matrix can also affect cell cycle progression and differentiation, at least in part by its ability to support cell spreading and a growth-permissive cytoskeletal organization. Adhesion to islands of ECM in which the spreading area is limited (Huang *et al.*, 1998) or adhesion to malleable ECMs that do not resist cytoskeletal tension (Mochitate *et al.*, 1991; Rosenfeldt and Grinnell, 2000; Wang *et al.*, 2000) will also inhibit progression through the G1 phase of the cell cycle, similar to loss of adhesion. Activation of PKA could result from a lack of resistance to cytoskeletal tension on collagen gel. Loss of tension in fibroblasts embedded within a mechanically loaded collagen matrices results in increased cAMP levels, loss of cytoskeletal organization, and disrupted growth factor signaling (Mochitate *et al.*, 1991; Rosenfeldt and Grinnell, 2000). A key similarity between these conditions and loss of adhesion is the disruption of cytoskeletal organization permissive for growth factor signaling. It is possible that a particular cytoskeletal organization, mediated by adhesion, plays a role in preventing inhibition of growth factor signaling by PKA, because PKA and its subunits associate with a number of proteins that anchor it to the actin cytoskeleton (Diviani and Scott, 2001). Rigid substrates that promote cell spreading and focal adhesion formation will typically support growth factor signaling. This is dependent on cytoskeletal integrity, because chemical disruption of the cytoskeleton on otherwise permissive ECM substrates will block cell cycle progression in G1 (Iwig *et al.*, 1995; Zhu and Assoian, 1995; Bohmer *et al.*, 1996; Bhadriraju and Hansen, 2004). Actin disruption by cytochalasin D treatment of cells blocks FAK activation in response to adhesion, but not integrin-dependent EGF receptor phosphorylation (Moro *et al.*, 2002). Furthermore, myosin-dependent contractility is required for G1 events such as ERK activation and cyclin D1 expression

even in noncontractile cells such as hepatocytes (Bhadriraju and Hansen, 2004). Thus, cytoskeletal integrity, including acto-myosin contractility, which are responsive to the mechanical nature of the ECM, play a key role in growth-permissive intracellular signaling and may be involved in the differential activation of PKA.

Although these results demonstrate that addition of collagen gel overlay can increase PKA activity and decrease DNA synthesis, it is not clear whether this is a result of switching from rigid collagen film adhesions to new adhesions formed between previously unoccupied integrins with compliant collagen adhesions or from activation of a collagen fibril-activated growth inhibitory receptor. In either case, adhesion to polymerized collagen can induce a dominant inhibitory signal that disrupts growth factor signaling toward G1-S progression. It has also been demonstrated *in vivo* that overexpression of type I collagenase in rat livers leads to cell proliferation (Nishio *et al.*, 2003), suggesting collagen may play a role in maintaining stasis under otherwise normal conditions. Whether PKA plays a role in this mechanism *in vivo* is not known. Because type I collagen is the most abundant protein in the human body, it will be important to determine the mechanism by which PKA is activated by adhesion to collagen fibrils and also to determine if PKA is activated in other cell types by type I collagen fibrils. Because PKA can promote cell proliferation in some cell types, while inhibiting the growth of others, the role of PKA in adhesion to type I collagen could be important for understanding fibrotic diseases, where type I collagen production increases and alters the architecture and cell population of the fibrotic tissue (Seyer *et al.*, 1977; Murata *et al.*, 1984). Because activation of PKA could be a result of biochemical or mechanical signaling from fibrillar collagen, understanding the mechanism by which PKA is activated will require separating the potential effects of fibril-specific ligands from the role of substrate malleability on PKA activity.

In conclusion, these results demonstrate that type I collagen structure plays a strong role in EGF signaling in hepatocytes. Data presented here demonstrate that this differential regulation of cell cycle progression is due at least in part to a novel ECM structure-dependent up-regulation of PKA activity and subsequent PKA-dependent alteration of EGF receptor phosphorylation and resulting inhibition of key G1 phase cell cycle events. These results could have important implications in understanding the role of adhesion to type I collagen fibrils in fibrotic diseases as well as in maintenance of homeostasis by type I collagen.

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