The Role of Collagen Structure in Mitogen Stimulation of ERK, Cyclin D1 Expression, and G1-S Progression in Rat Hepatocytes*

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Adhesion to type 1 collagen can elicit different cellular responses dependent upon whether the collagen is in a fibrillar form (gel) or monomeric form (film). Hepatocytes adherent to collagen film spread extensively, express cyclin D1, and increase DNA synthesis in response to epidermal growth factor, whereas hepatocytes adherent to collagen gel have increased differentiated function, but lower DNA synthesis. The signaling mechanisms by which different forms of type I collagen modulate cell cycle progression are unknown. When ERK MAP kinase activation was analyzed in hepatocytes attached to collagen film, two peaks of ERK activity were demonstrated. Only the second peak, which correlated with an increase of cyclin D1, was required for G1-S progression. Notably, this second peak of ERK activity was absent in cells adherent to collagen gel, but not required in the presence of exogenous cyclin D1. Expression of activated mutants of the Ras/Raf/MEK signaling pathway in cells adherent to collagen gel restored ERK phosphorylation and DNA synthesis, but differentially affected cell shape. Although Ras, Raf, and MEK all increased expression of cyclin D1 on collagen film, only Ras and Raf significantly up-regulated cyclin D1 levels on collagen gel. These results demonstrate that adhesion to polymerized collagen induces growth arrest by inhibiting the Ras/ERK-signaling pathway to cyclin D1 required in late G1.

The extracellular matrix (ECM) provides a variety of biochemical and mechanical signals that profoundly affect cell behavior. Adhesion to certain types of ECM can promote cell-specific responses including differentiation or proliferation, whereas loss of adhesion results in growth arrest in mid-G1 of the cell cycle (1–3). Whereas adhesion is generally required for cell cycle progression, adhesion to certain ECMS can inhibit cell cycle progression and/or increase differentiated function. Little is known, however, about the mechanisms of cell cycle arrest by these growth inhibitory substrates.

Recently mechanisms have been identified that mediate adhesion-dependent cell cycle progression. A major target of cooperative signaling from integrins and growth factor receptors is the expression of cyclins, which bind to and activate cyclin-dependent kinases (CDKs), forming active kinase complexes required for progression through specific points in the cell cycle. G1 arrest in response to loss of adhesion is often characterized by lower levels of cyclin D1 (4, 5) and/or higher levels of the cyclin-dependent kinase inhibitors (CKIs) p21 (1) or p27 (1, 2), and exogenous expression of cyclin D1 can restore G1-S progression in the absence of adhesion in some cell types (2, 4, 6). Expression of cyclins and CKIs is regulated by growth factor receptor tyrosine kinase-dependent signal transduction pathways that require integrin-mediated adhesion for maximal activity (7). The Ras/Raf/MEK/ERK signaling cascade lies downstream of both growth factor receptor tyrosine kinase activation and integrin activation (8–10). Sustained activation of this pathway is important for cyclin D1 expression and G1-S phase progression in fibroblasts (8, 11), and loss of adhesion inhibits growth factor signaling through this pathway and blocks G1-S progression (9, 10, 12).

Both mechanical and biochemical properties of the ECM are conveyed through integrins, and these properties affect different responses in activation of intracellular signaling pathways and/or morphogenic changes in the cell. For instance, endothelial cell adhesion to laminin via α5β1 integrin subunits decreases cyclin D1 translation and inhibits cell cycle progression, whereas adhesion to fibronectin via αvβ1 promotes cyclin D1 expression and cell cycle progression (13). Also, fibroblasts embedded within polymerized collagen fail to progress through the cell cycle when tension is released from the matrix. This failure is associated with decreased activation of ERK, decreased expression of cyclin D1, and increased expression of p27 (14). Thus, both biochemical and mechanical properties of the ECM are important for integration of growth factor signaling into the cell cycle machinery. Because most cells in vivo are in constant contact with the ECM, and the mechanical and biochemical properties of the ECM in vivo are dramatically different from rigid plastic dishes often used in cell culture, understanding how growth factor signaling is regulated by more physiological forms of ECM will be important for understanding cell behavior during disease and development.

Primary cultures of hepatocytes provide a physiologically relevant model to study the signal transduction mechanisms by which different ECMS regulate cell cycle progression and differentiation. Although quiescent under normal conditions, hepatocytes in the liver rapidly enter and proceed through the

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† The abbreviations used are: ECM, extracellular matrix; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; EGF, epidermal growth factor; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; CMV, cytomegalovirus; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; CA-MEK, constitutively activated MEK.
Collagen Regulation of ERK and Cyclin D1 in Hepatocytes

cell cycle after chemical injury or partial hepatectomy in a fairly synchronous manner to restore liver mass and function (15–17). This response is believed to be stimulated in part by the release of cytokines and growth factors including hepatocyte growth factor, EGF, and transforming growth factor α (15, 17), and can be mimicked in primary cultures of hepatocytes treated with growth factors (18–20). Mitogenic signaling through the Ras/Raf/MEK/ERK cascade (20–22) and cyclin D1 expression (23–25) have been demonstrated to play a role in hepatocyte cell cycle progression, and recently MEK has also been shown to play a morphogenic role in hepatocytes attached to tissue culture plastic (20). Whereas a number of studies have investigated growth factor-mediated signal transduction pathways and cell cycle progression in hepatocytes, there has been less investigation into how signaling is modulated in hepatocytes by the ECM. As adhesion and growth factors play a concerted role in signal transduction to G1-S progression (26), elucidating the regulation of hepatocyte cell cycle progression in the context of signaling from both growth factors and adhesion is necessary.

Type I collagen is the most abundant protein in the human body and a major protein component of extracellular matrix. Its importance in normal physiology is illustrated by the numerous diseases that result from mutations in collagen (27) or from genetic knockouts of adhesion receptors that bind collagen (28, 29). Interaction with type I collagen may be particularly relevant in liver fibrosis and cirrhosis, where type I collagen fibrils increase (30–32), eventually leading to a decrease in proliferative response. In cultured hepatocytes (24, 33–35) and other cells, including mammary epithelial cells (36, 37), keratinocytes (38, 39), and endothelial cells (40), polymerized collagen has been used as a substrate upon which to elicit differentiated function and long term survival. Whereas differentiated function increases in hepatocytes adherent to polymerized collagen, hepatocyte proliferation is often diminished, despite adhesion to the ECM and the presence of mitogens that normally induce proliferation on monomeric collagen (24, 41, 42). The mechanism, however, by which adhesion to polymerized collagen prevents growth factor signaling to G1-S progression and/or promotes differentiation is not known.

Studies from this laboratory and others have previously demonstrated that adhesion to polymerized collagen (gel) inhibits hepatocyte cell spreading and cell cycle progression (24, 41), while adhesion to monomeric collagen (film) allows cell spreading, robust cyclin D1 expression, and permits G1-S progression in response to EGF. The signal transduction mechanisms by which differences in collagen structure regulate hepatocyte cell shape, cell cycle protein expression, and G1-S progression have not yet been elucidated. The purpose of the study described below was to identify the mechanisms by which adhesion to collagen gel inhibits growth factor signaling and cell cycle progression. We report that adhesion to different forms of type I collagen regulates signaling through the Ras/Raf/MEK/ERK cascade, and that individual components of this pathway play distinct and dynamic roles in hepatocyte cell shape, cyclin D1 expression, and G1-S progression.

MATERIALS AND METHODS

Reagents—The cyclin D1 adenovirus was previously described (24). Adenovirus expressing constitutively activated MEK (CA-MEK; S218E/S222D), membrane-targeted Raf (bax-Raf), Raf (V12), and Raf (N17) were kindly provided by Dr. Luis F. Parada (University of Texas, Southwestern) (45). The 1745-base pair cyclin D1 promoter-driven luciferase gene was kindly provided by Dr. Richard Postell (Albert Einstein Comprehensive Cancer Center) (44). The albumin promoter-luciferase construct was kindly provided by Dr. Gretchen J. Darlington (Baylor Medical College) (45). U0126 was from Promega (Madison, WI).

Hepatocyte Culture—Primary rat hepatocytes were obtained by collagenase perfusion of adult Lewis rat liver (46), followed by purification using Percoll gradient (Sigma). Only cells from harvests yielding >90% viability were used. Hepatocytes were plated at subconfluent density (10,000–12,000 cells/cm²) in serum-free William’s medium E (Invitrogen) with the following additives as previously described (24): epidermal growth factor (10 ng/ml, Collaborative Research, Bedford, MA), insulin (20 milliunits/ml, Sigma), dexamethasone (5 nM, Sigma), sodium pyruvate (1 mM, Invitrogen), ascorbic acid (50 μg/ml, Invitrogen), and penicillin/streptomycin (100 units/ml, Irvine Scientific, Santa Ana, CA). Cultures were re-fed daily. Cultures were always performed in the presence of growth factors unless otherwise stated.

Collagen Substrate Preparation—Type I collagen (“Vitrogen 100,” Collagen Biomaterials, Palo Alto, CA) was coated onto non-adhesive polystyrene dishes to produce either monomeric collagen film or, a polymerized form referred to as collagen gel. Collagen film is prepared by coating dishes with collagen diluted in a basic, carbonate buffer (pH 9.4) as previously described (24). To produce a collagen gel substrate, 50 μl of NaOH was added to each milliliter of 5% William’s E medium. 1 part of 5% William’s E medium, then adding to adherent cells. New media was added after the overlay had gelled (~4 h).

DNA Synthesis—DNA synthesis is measured by adding 3[H]thymidine (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 and Western Blot—Cultured hepatocytes were removed from the plates by scraping in lysis buffer as previously described (25). Western blot analysis was performed using ~30 μg of cellular protein per lane as previously described (25). A loading control was obtained by stripping and reprobing blots with mouse anti-glycerol-3-phosphate dehydrogenase (GAPDH). Antibodies used for Western blots include anti-rat cyclin E (catalog number sc-481, Santa Cruz Biotechnology, Santa Cruz, CA), anti-active ERK and anti-pan-ERK (catalog numbers 9102 and 9101, respectively, Cell Signaling, Beverly, MA), anti-cyclin D1 (catalog number 6B-17, Upstate Biotechnology, Lake Placid, NY), and anti-GAPDH (catalog number ab8245–100; Abcam Limited, Cambridge, UK). Western blots were visualized using ECL-plus reagent (Amersham Biosciences), and band intensity was quantitated using STORM imaging and Amersham Biosciences software (Amersham Biosciences), and model GS-700 imaging densitometer and molecular analyst software (Bio-Rad). Statistical comparisons were performed using the t test.

Adenovirus Transfection—Rat hepatocytes were infected with adenovirus 3–6 h after plating, as previously described (24). Approximately 10 “TCID 50” units of adenovirus were used per cell as previously described (24). Adenovirus expressing the β-galactosidase or green fluorescent protein (GFP) was used at the equivalent titer as a control.

Plasmid Transfections and Promoter Activity Luciferase Assays—Promoter-luciferase plasmids were purified using an endotoxin-free maxi-kit (Qiagen, Valencia, CA). For experiments on collagen film, hepatocytes were plated in William’s E medium with EGF at 1 × 10⁵ cells/35-mm dish and transfection 6–12 h after plating using FuGENE HD (Roche Diagnostics). As described by the manufacturer, this luciferase plasmids were routinely co-transfected with control plasmids expressing Renilla luciferase under the control of either a null or CMV promoter (Null Renilla, or CMV Renilla; Promega). Transfection on collagen gel resulted in very low transfection efficiency. To compare film and gel conditions, 2 × 10⁵ hepatocytes were transfected on a 10-cm collagen-coated dish, then plated onto 35-mm dishes coated with 1 ul of film or gel. Briefly, 300 μl of William’s E medium (no additives) containing 9 μl of FuGENE reagent was mixed with 3 μg of plasmid DNA, incubation 10 min at room temperature, then added to cells for 6–12 h. After a 6–12-h incubation with plasmids/FuGENE, cells were washed twice in PBS without Ca²⁺ or Mg²⁺, then trypsinized and replated onto 35-mm dishes coated with 1 ul of gel or film. 1 ul of null or CMV promoter activity, hepatocytes adherent to 35-mm dishes of collagen film were washed twice in PBS, then lysed in 100 μl of 1× dual luciferase lysis buffer as described (Promega). Cells adherent to collagen gel were detached from the matrix with 1 mg/ml collagenase (Roche Diagnostics).
in PBS, then centrifuged and washed twice in PBS before adding 100 μl of lysis buffer. The differences in harvesting methods had no detectable effect on either firefly or Renilla luciferase activity. Unless otherwise stated, lysates were harvested at 72 h and frozen at −70 °C. Luciferase activity was measured using the dual luciferase assay kit (Promega) and normalized to Renilla values within the same lysate.

In Vitro Kinase Assay—Kinase assays were performed as previously described (47). Briefly, cell lysates were immunoprecipitated with anti-ERK antibody followed by kinase assay in which immunobead complexes were incubated for 30 min with kinase buffer containing 5 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham Biosciences) plus substrate (myelin basic protein). Reaction was terminated by addition of 2× SDS-PAGE sample buffer, and samples were analyzed by 12% SDS-PAGE and phosphorimaging.

Cell Size Measurements—Hepatocytes were cultured for 48 h, fixed with 4% paraformaldehyde, washed 3 times in PBS, then stained for 5 min with Coomassie Blue/methanol solution. Cells were then washed five times in PBS and measured using computerized image analysis (Image Pro Plus, Carlsbad, CA).

**RESULTS**

**Cyclin D1 Protein Levels and Promoter Activity Are Inhibited by Adhesion to Collagen Gel**—It has been previously demonstrated that increased cyclin D1 in hepatocytes during mid to late G1 is dependent on stimulation by mitogens such as EGF and insulin (23, 25). Furthermore, cyclin D1 expression is inhibited in hepatocytes adherent to low density collagen2 or denatured collagen fibrils even in the presence of EGF and insulin (24). Similarly, culture of hepatocytes on collagen gel with growth factors results in a lower level of cyclin D1 protein expression than culture on a film of monomeric collagen (Fig. 1A). Cyclin D1 expression is regulated at the transcriptional as well as post-transcriptional levels (44, 48–50). To determine at what level cyclin D1 expression was regulated by adhesion to different collagen substrates, the transcriptional activity of a 1745-bp human cyclin D1 promoter construct fused to the luciferase gene was measured under different ECM conditions. As shown in Fig. 1B, the activity of the 1745-bp human cyclin D1 promoter is up-regulated in a similar time course to the previously described up-regulation of cyclin D1 mRNA in primary hepatocytes (18), demonstrating a large increase in activity between 48 and 72 h after plating. This increase in cyclin D1 expression correlates with entry into S phase, which occurs 42–52 h after plating. To determine how the interaction with polymerized collagen affects cyclin D1 transcription, the 1745-bp cyclin D1 promoter activity was measured in hepatocytes adherent to film or gel. Because the plasmid transfection efficiency on gel was insufficient to measure luciferase activity, cells were transfected on film and then trypsinized 6–12 h later and replated on film or gel, and luciferase activity was measured 48 h after replating. As demonstrated in Fig. 1C, culture of hepatocytes on collagen gel decreases the relative activity of the cyclin promoter to 30% of that found on film. Similar results were demonstrated using a 1810-bp rat cyclin D1 promoter (data not shown). The higher activity on film suggests that cyclin D1 gene expression is transcriptionally regulated in hepatocytes by adhesion to different forms of type I collagen.

**Adhesion to Collagen Gel Inhibits G1-S Progression**—To determine how the loss of cyclin D1 expression on collagen gel affected G1-S progression, DNA synthesis was measured in cells adherent to collagen film or collagen gel. As shown (Fig. 2), adhesion to collagen gel substantially diminished DNA synthesis to the low level observed in the absence of growth factors on film. However, infection with an adenovirus vector, which yields high transfection efficiency in hepatocytes (24), expressing a human cyclin D1 gene under control of the CMV promoter completely restores DNA synthesis both in the absence of growth factors as previously shown (25) as well as in hepatocytes growth-arrested on collagen gel. Restoration of DNA synthesis by exogenous cyclin D1 transcription suggests that cyclin D1 is a limiting factor in hepatocyte cell cycle progression, and that regulation of cell cycle progression by collagen gel occurs upstream of cyclin D1 transcription.

To determine whether adhesion to collagen gel was actively inhibiting cyclin D1 promoter activity (that is, providing a signal that inhibits promoter activity) or passively preventing promoter activity (i.e. lacking a stimulatory signal normally provided by adhesion to collagen film), hepatocytes were plated onto collagen film and transfected with the cyclin D1 promoter-driven luciferase gene or an albumin promoter-driven luciferase gene, then treated 18 h after plating with a collagen gel overlay. Cyclin D1 promoter activity was dramatically dimin-

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2 L. K. Hansen, unpublished data.
lished after overlay treatment (Fig. 3A), demonstrating that collagen gel actively inhibits cyclin D1 expression despite attachment to a permissive collagen film substrate. The albumin promoter activity was not significantly altered, showing that diminished cyclin D1 promoter activity was because of specific inhibition by hepatocyte interaction with collagen gel and not because of an overall decrease in gene expression. Similar to the effects on the cyclin D1 promoter, addition of collagen gel overlay also decreased G1-S progression when added prior to S phase (Fig. 3B). When collagen gel overlay was added early in the culture, the inhibition of DNA synthesis was greater than when it was added after the restriction point (around 42–48 h (25)), suggesting that adhesion to collagen gel inhibits cell cycle progression in mid-G1 despite the presence of EGF. Thus, adhesion to type I collagen gel actively inhibits transcription of cyclin D1 and subsequent hepatocyte cell cycle progression.

EGF-induced p44/42 ERK Activity Is Diminished on Collagen Gel—Previous evidence suggests that expression of the cyclin D1 gene is regulated by p44/42 ERK (ERK 1/2) signaling (51). To investigate the role that adhesion to collagen gel plays in p44/42 ERK activation, activation of these enzymes was analyzed in hepatocytes cultured on film or gel continuously in the presence of insulin and EGF for the indicated times (Fig. 4A). A dramatic difference in activation of p44/42 ERK is demonstrated between cells attached to film or gel. Although p44/42 ERK activity appears similar on both substrates at 2 h, the second peak of p44/42 ERK activation shown on collagen film is absent on the collagen gel. It should be noted that p44 ERK is phosphorylated in freshly isolated hepatocytes prior to plating. Our unpublished data and that of others (52) suggests that early activation of ERK and G0-G1 progression occurs during liver perfusion itself.

To quantitate the expression level of ERK, cyclin D1, and cyclin E, densitometry was performed and normalized to

3 L. Jungers and L. K. Hansen, unpublished data.
Collagen Regulation of ERK and Cyclin D1 in Hepatocytes

Fig. 5. Cyclin D1 expression and G1-S progression require MEK activity in late G1 and S phase. A, hepatocytes adherent to collagen film were treated with different doses of U0126 or Me2SO (vehicle) beginning at 30 h after plating, and DNA synthesis was measured by [3H]thymidine incorporation between 48 and 72 h. Data represents average and S.D. of triplicate samples (** indicates p < 0.01 compared with Me2SO control). B, hepatocytes were treated with 25 μM U0126 or equivalent Me2SO for the indicated 24-h periods, and DNA synthesis was measured by [3H]thymidine incorporation between 48 and 72 h. Data are average and S.D. of four independent experiments each done in triplicate cultures (** indicates p < 0.01 compared with Me2SO control). C, hepatocytes were treated from 30 to 48 h with different doses of U0126, at which time lysates were collected and analyzed for cyclin D1 expression by Western blot. D, hepatocytes were transfected with the human 1745-bp cyclin D1 promoter and treated with 25 μM U0126 as above. Lysates were analyzed for firefly luciferase activity relative to co-transfected Renilla luciferase activity at 72 h (** indicates p < 0.01 compared with Me2SO control).

Inhibition of MEK Blocks EGF-induced Cyclin D1 Expression and DNA Synthesis on Collagen Film—MEK is the upstream kinase that activates p44/42 ERK. While strong evidence suggests that MEK regulates cyclin D1 expression and G1-S progression in EGF-stimulated hepatocytes (20, 21), there are also contradictory reports regarding the role of this pathway (22, 53). However, the report demonstrating a critical role for MEK in hepatocyte cell cycle progression used tissue culture-treated plastic for hepatocyte culture rather than a defined ECM (21). Therefore, the role of MEK was investigated in EGF-stimulated hepatocyte cell cycle progression and cyclin D1 expression in hepatocytes adherent to a film of monomeric type I collagen. DNA synthesis was measured after blocking the second peak of MEK with U0126, a specific inhibitor that blocks both activation of MEK as well as the activity of MEK (54). Addition of U0126 at 30 h of incubation, which is prior to the second peak of ERK activation, inhibits DNA synthesis in a dose-dependent fashion (Fig. 5A).

Because the activity of ERK appears to be regulated in a biphasic fashion during progression through G1, the temporal role of MEK in G1-S progression was examined at different times throughout the culture period. Treatment of hepatocytes with 25 μM U0126 at different time points after plating revealed that inhibition of the MEK/ERK pathway during the first 48 h has little effect on DNA synthesis measured from 48 to 72 h, but treatment at 48 h significantly inhibited G1-S progression (Fig. 5B). Curiously, this point of particular sensitivity to MEK inhibition is around the G1-S restriction point (18), at which time EGF is no longer necessary for progression through the S phase (25). To examine the effect of MEK inhibition on cyclin D1 expression, cells were treated with different concentrations of U0126 beginning at 30 h of culture, and cyclin D1 expression was measured at 48 h after plating. U0126 treatment blocked cyclin D1 expression in a dose-dependent fashion similar to its effect on DNA synthesis, demonstrating that cyclin D1 and G1-S progression are dependent upon MEK activity during late G1 and S phase (Fig. 5C). The cyclin D1 promoter also appeared to be regulated by MEK, as inhibition of MEK decreased activity of the cyclin D1 promoter relative to co-transfected CMV promoter activity (Fig. 5D). Together, these results suggest that MEK activity is important for cyclin D1 expression, and that on a permissive ECM, this activity is only necessary during late G1 and S phase.

The results presented so far suggest that MEK activity regulates cyclin D1 transcription and progression into S phase in cells adherent to collagen film. To determine whether MEK has a critical role in G1-S progression besides increasing cyclin D1 expression, and to rule out a nonspecific effect of U0126 on cell viability, hepatocytes were infected from 3 to 6 h with adeno-virus expressing cyclin D1 or a control protein (GFP), and MEK was inhibited by addition of 25 μM U0126 at 30 h after plating. Exogenous cyclin D1 expression counteracted the inhibition of DNA synthesis in cells treated with 25 μM U0126, whereas DNA synthesis in control adenovirus-infected cells was diminished (Fig. 6). U0126 also appears to decrease DNA synthesis in cells expressing cyclin D1 somewhat, although not to the significant extent that control adenovirus-expressing cells were inhibited. These results demonstrate for the first time that MEK activity is not required for progression into S phase in the presence of exogenous cyclin D1 and they suggest that the...
major contribution toward $G_1$-S phase progression in hepatocytes provided by MEK is the up-regulation of the cyclin D1 gene.

Activation of Ras Pathway Components Differentially Affects Cell Spreading, Cyclin D1 Expression, and G1-S Progression on Film or Gel—Because it appeared that EGF signaling to ERK is disrupted by adhesion to polymerized collagen, it was of interest to investigate at what point the disruption occurred. Therefore, the canonical upstream members of the signaling cascade to ERK (Ras, Raf, and MEK) were tested for the ability to restore $G_1$-S progression in hepatocytes adherent to collagen gel. Infection of hepatocytes with adenovirus expressing activated forms of Ras(V12), membrane-targeted Raf(bxb), or constitutively active MEK(S218E/S222D) (43) shortly after plating resulted in visible differences in cell shape on collagen gel by 48 h (Fig. 7, A, photomicrographs; B, cell area measurements). Ras(V12) increased spreading the most, whereas activated Raf and activated MEK had only a slight effect on spreading on collagen gel. These results suggest that the lack of spreading on the gel is not because of the malleable nature of the substrate, but rather to the inhibition of intracellular signaling pathways controlling cell spreading.

To determine whether expression of activated forms of Ras pathway mutants were sufficient to restore ERK activation and cyclin D1 expression on collagen gel, lysates from ras(V12), bxb $raf_1$, and CA-MEK-transfected cells adherent to collagen film or collagen gel were examined for ERK phosphorylation, as well as cyclin D1 protein expression in hepatocytes on collagen gel, with Raf inducing a moderate but significant increase. MEK and Raf are sufficient to induce ERK phosphorylation on collagen gel, they are not sufficient for high levels of cyclin D1 phosphorylated in proliferating hepatocytes on collagen film, and this pool can be activated by constitutive activation of the Ras pathway components.

Each of the Ras pathway mutants was also able to increase cyclin D1 protein expression in hepatocytes on collagen gel, however, each to a different extent (Fig. 8, A and B). Ras(V12) was most effective at increasing cyclin D1 expression on collagen gel, with Raf inducing a moderate but significant increase. CA-MEK demonstrated a nominal and non-significant induction of cyclin D1. In cyclin D1 promoter assays, only Ras completely overcame inhibition of cyclin D1 transcription by adhesion to collagen gel (Fig. 8C). These results suggest that while MEK and Raf are sufficient to induce ERK phosphorylation on collagen gel, they are not sufficient for high levels of cyclin D1 induction in the absence of additional signal(s) provided by adhesion to collagen film or active Ras.

On collagen gel, all three active mutants increased cyclin D1 expression to much higher levels than GFP-transfected hepatocytes or non-transfected hepatocytes on film, similar to their effects on ERK phosphorylation. Cyclin E expression was not significantly altered by addition of the activated Ras pathway mutants on collagen film, but there was a small induction on collagen gel. Dominant negative Ras mutant (Ras(N17)) consistently inhibited ERK activation, cyclin D1, and cyclin E expression on collagen gel, emphasizing the requirement of Ras for induction of these cell cycle events.

To examine the effects of the Ras/Raf/MEK components on entry into S phase in hepatocytes adherent to collagen gel, DNA synthesis was measured in transfected hepatocytes. In cells adherent to gel, Ras, Raf, and MEK induced DNA synthesis (measured from 12 to 72 h) significantly higher than that seen on GFP controls on gel (Fig. 9A). Similar to their effects on cyclin D1 protein, ras and raf appeared more effective than...
MEK at inducing DNA synthesis on the collagen gel, but each one still induced DNA synthesis to levels seen in proliferating cells on collagen film (GFP controls). Furthermore, each Ras pathway mutant, as well as cyclin D1 adenovirus, significantly increased DNA synthesis in hepatocytes on collagen film well above that observed in film-plated GFP controls, whereas dominant negative Ras(N17) decreased total DNA synthesis, consistent with its inhibition of cyclin D1 expression.

Whereas the peak of DNA synthesis on collagen film in control cells is between 48 and 60 h, activated Ras, Raf, or MEK resulted in more rapid entry into S phase in cells on film, beginning between 24 and 36 h, and peaking at 36 to 48 h after plating (Fig. 9B). Dominant negative Ras, however, particularly inhibited DNA synthesis between 48 and 72 h. In contrast, the active ras mutants induce a time course of DNA synthesis on gel that is comparable with control cells on film. Whereas Ras and Raf equally overcame growth arrest on collagen gel, activated MEK was less efficient at restoring DNA synthesis on gel, even though it was very effective at increasing DNA synthesis on collagen film above the already high levels observed in controls. These results demonstrate that Ras, Raf, and MEK differ in the ability and mechanism by which they overcome growth arrest in response to adhesion to different forms of collagen.

**DISCUSSION**

Results presented here demonstrate that despite adhesion to the ECM and the presence of mitogens, which are both requirements for cell cycle progression in anchorage-dependent cells, cell cycle progression is strongly dependent upon the structure of the ECM. Investigation into the signal transduction pathways involved in the different response to monomeric collagen film and polymerized collagen gel has revealed important insights into the role of the Ras/Raf/MEK/ERK pathway, cyclin D1 expression, and cell shape in hepatocyte cell cycle progression. Most significant is the observation that adhesion to polymerized collagen blocks EGF signaling to ERK and cyclin D1 expression, which prompted us to further define the role of collagen structure in modulating the Ras signaling pathway to cyclin D1 expression on different collagen substrates.
Contradictory reports regarding the role of the Ras/MEK/ERK signaling pathway in hepatocyte cell cycle progression led to the need to first establish the role of MEK in hepatocyte cell cycle progression on collagen film. In primary hepatocytes, the p44/42 ERK MAP kinase pathway has previously been implicated as being important for G₁-S progression in some reports (20, 21, 55), while having no role in a separate report, in which a role for phosphatidylinositol 3-kinase is implicated independent of MEK (53). Another report suggested that sustained activation of the ERK pathway by Raf inhibits hepatocyte DNA synthesis, while transient activation of this pathway stimulates DNA synthesis (22). Our data demonstrates that MEK plays a key role in cyclin D1 expression and hepatocyte G₁-S progression specifically in late G₁ and S phase. ERK activation occurs in two peaks on collagen film in response to EGF. The first peak begins during hepatocyte isolation from the liver, as demonstrated by fairly high phosphorylation levels relative to total ERK protein in freshly isolated cells. In the absence of EGF this first peak is rapidly lost (20), whereas in EGF-stimulated cells on monomeric collagen, it is sustained throughout the culture period with a decrease around 12 to 36 h, after which a second peak develops that is sustained during S phase as well. The biphasic activation of ERK demonstrated in hepatocytes adherent to collagen film is similar to a biphasic activation of ERK during liver regeneration, but that occurs during a much shorter time course (21). It is this second peak of ERK activation that is important for cyclin D1 expression and G₁-S progression in cultured hepatocytes as inhibition of MEK at this time was most effective at inhibiting increased cyclin D1 levels and cell cycle progression. Exogenous expression of cyclin D1, however, restored DNA synthesis in MEK-inhibited cells, suggesting that the major role for MEK in hepatocyte cell cycle progression on a permissive matrix is increasing cyclin D1 expression, and MEK activity is not required for cell cycle progression in the presence of adequate levels of cyclin D1.

It was also demonstrated that inhibition of MEK prior to 48 h had little effect on DNA synthesis between 48 and 72 h, suggesting that in cells adherent to a permissive ECM, MEK is not required prior to the restriction point. Whereas a recent role for early G₁ MEK activity in hepatocyte spreading on tissue culture plastic has been described (20), this was not evident in our hands when the cells were cultured on collagen. We noted however, found that blocking MEK activity does inhibit spreading when cells were cultured on tissue culture plastic, suggesting that the early role of MEK in spreading may only be apparent when cells are dependent upon MEK to produce their own matrix on which to spread (20). Whereas MEK may not be required during early and mid G₁, EGF stimulation is required, as hepatocytes cultured in serum-free media without EGF for the first 48 h fail to enter S phase between 48 and 72 h when EGF is added at 48 h. This demonstrates that the early G₁ role of EGF in cells adherent to a permissive substrate is independent of MEK, but that progression through the restriction point and into S phase requires MEK.

The lack of late G₁ ERK phosphorylation, cyclin D1 expression, and DNA synthesis on collagen gel in the presence of EGF indicated that adhesion to collagen gel inhibits EGF-dependent signals upstream of ERK. Although integrin-mediated binding to fibronectin or type I collagen potentiates growth factor signaling toward cell cycle progression (56), and lack of adhesion inhibits growth factor signaling (4, 7), less is known about how certain ECMs depress growth factor signaling. Results from the collagen gel overlay experiments suggest that this occurs through activation of an inhibitory signal rather than simply a lack of the positive signal(s) induced on film. However, the nature of the negative signal(s) and the mechanism of induction is not clear. One possibility is that different receptors are used for adhesion to different forms of collagen, and differential use of particular integrin subunits alters growth factor signaling pathways. In smooth muscle cells, adhesion to fibrillar collagen prevents G₁-S progression through α₁ integrin-dependent up-regulation of p21 and p27 (57). In endothelial cells, translation of cyclin D1 and G₁-S progression is inhibited by adhesion to laminin via α₁β₁, but permitted by adhesion to fibronectin via α₅β₁ (13). In hepatocytes, adhesion to collagen gel is mediated by α₁β₁ integrin (58). It is possible, however, that other integrin α subunits that block growth factor signaling are involved in adhesion to polymerized collagen gel. Primary hepatocytes do not express α₂ subunits (58), however, it has been reported that the α₂ subunit is expressed in primary hepatocytes (58) and expression of this subunit in a conditionally transformed mouse hepatocyte cell line is involved in the differentiated response to collagen gel (59). Investigation of this and other α subunits that may be involved in adhesion specifically to the collagen gel is an area currently under investigation. Another possibility is that non-integrin receptors specific for polymerized collagen play a role in inhibition of growth factor signaling. The identification of the DDR2 tyrosine kinase receptor, which is specifically activated by polymerized collagen (60), further suggests that organisms have evolved mechanisms to specifically respond to different forms of collagen. It is conceivable that a receptor such as this could activate an inhibitory pathway antagonistic to EGF signaling, although the details of downstream signaling pathways from this receptor are not currently known.

Whereas activation of different integrin subunits may play a role in growth factor signaling, the mechanical and physical properties of the matrix have also been shown to play a role in regulating G₁-S progression. In fibroblasts, if the mechanical properties of the matrix are altered so there is less resistance within the matrix to contractile forces, then the cells contract the gel, attain a round shape, and demonstrate diminished ERK activation, cyclin D1 expression, and G₁-S progression (14). As well, altering the physical boundaries of the matrix by limiting the area of ECM ligands, such that adhesion is allowed but spreading is inhibited, also limits G₁-S phase progression in hepatocytes (35, 61) or endothelial cells (40, 62). In endothelial cells unable to spread, inhibition of G₁-S occurs despite ERK activation, and involves increased expression of p27 and decreased cyclin D1 protein levels (63). We have found that adhesion to polymerized collagen prevents cell spreading and this decrease is associated with lower levels of cyclin D1 expression, but this decrease appears to be transcriptionally regulated, whereas inhibition of endothelial cell spreading blocked cyclin D1 expression at the level of translation. When collagen was allowed to polymerize over hepatocytes, allowing adhesion to a permissive substrate on the basal cell surface, and interaction with an inhibitory substrate on the upper surface, cyclin D1 transcription and DNA synthesis were blocked. This is further evidence that inhibition of cyclin D1 expression by adhesion to polymerized collagen is because of an interaction with polymerized collagen that actively inhibits G₁-S progression, rather than lack of a permissive interaction found on monomeric collagen that helps drive progression into S phase. In collagen gel overlays, hepatocytes begin to adhere to overlaid collagen and form fewer new adhesions with the basal layer of monomeric collagen at the cell borders (data not shown). This results in a more rounded cell shape, despite continued adherence to the monomeric collagen film on the basal surface. A common theme among these examples and many others in the

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4 J. T. Fassett and L. K. Hansen, unpublished data.
literature is that a spread cell shape and/or the ability to generate cytoskeletal tension at adhesion sites is associated with progression into S phase, whereas inhibition of spreading prevents DNA synthesis.

Because hepatocyte spreading was diminished on collagen gel, it was of interest to determine at what point(s) along the EGF signaling cascade adhesion to collagen gel and, potentially, the differences in cell shape blocked signaling to ERK, cyclin D1 expression, and G1-S progression. When activated Ras, Raf, and MEK were expressed in hepatocytes, each restored ERK phosphorylation and G1-S on collagen gel, suggesting that disruption of signaling to ERK on the collagen gel occurs either upstream of Ras or is eliminated by activated Ras. Furthermore, dominant negative Ras completely blocked ERK activation at 48 h, suggesting that Ras plays an important role in signaling to ERK in hepatocytes.

Expression of activated mutants of Ras, Raf, and MEK also differentially affected cell morphology. Ras(V12) caused extensive cell spreading on the collagen gel, whereas activated forms of Raf and MEK did not significantly increase the cell area. This suggests, however, that inhibition of spreading on the collagen gel is not simply because of a mechanical property of the gel, but the result of a specific pathway that prevents cell spreading and that is overridden or avoided by re-activation of the Ras signaling pathway. Recently it has been demonstrated that activated Ras and the downstream kinase MEK inhibits expression of Rho kinase, contributing to the morphological transformation of cells expressing active Ras (64). As Rho and its effector Rho kinase drive stress fiber formation and cytoskeletal contraction (65), it is possible that Rho stimulates contraction of the collagen matrix, resulting in cell rounding. We have found, however, that chemical inhibition of MEK from 24 to 48 h (added prior to cell spreading on gel in Ras pathway mutant-transfected cells), had little effect on spreading in hepatocytes expressing Ras, Raf, or MEK on collagen gel, suggesting that other mechanisms may also be responsible for inducing cell spreading on collagen gel. Furthermore, we have found that inhibition of Rho using C3 exotoxin blocks cyclin D1 expression in hepatocytes adherent to collagen film (24). Whereas the precise mechanism by which re-activation of Ras signaling induces spreading remains to be determined, the demonstration that activated MEK, Raf, or Ras restored ERK activation and that ras increases spreading suggests that inhibition of this pathway on the collagen gel likely occurs upstream of or at the level of Ras activation.

Curiously, activated Ras, Raf, and MEK each dramatically increased cyclin D1 expression in EGF-treated cells on collagen film, but on collagen gel, Ras was significantly more effective at inducing cyclin D1 than Raf or MEK. Stronger induction of cyclin D1 on collagen gel by Ras was observed even though there was no significant gel-induced inhibition of ERK activity in Ras, Raf, or MEK-transfected hepatocytes. In fact, whereas activated MEK induced ERK activity on gel to levels equal or greater than non-transfected cells on film, the expression of cyclin D1 on MEK-transfected cells was ~10% that of cells on film. These results, combined with the results using U0126, suggest that while MEK may be important for cyclin D1 expression on permissive substrates, it is not sufficient to induce cyclin D1 on polymerized collagen. Evidence showing that activation of ERK in suspended CCL9 cells fails to activate cyclin D1 expression have suggested that other adhesion-dependent signals in addition to ERK may be required in some cell types to induce cyclin D1 (66). One potential mechanism by which ERK is prevented from signaling to cyclin D1 on collagen gel, suggested by the vastly different cell shapes on these substrates, might be cytoskeletal differences between cells adherent to collagen film or collagen gels and the ability to form intracellular cytoskeletal scaffolds important for interaction between signaling proteins. It has also been demonstrated that suspended fibroblasts or fibroblasts with disrupted cytoskeletons fail to translocate activated ERK to the nucleus, whereas cyclin D1 nuclear localization is not affected (10). Whereas cytoskeletal effects on ERK translocation could explain some of the differences observed in the ability of MEK to activate cyclin D1 expression on film or gel, this is not likely, because activating MEK or Raf still causes progression into S phase, suggesting that this pathway still induces nuclear events required for progression into S phase. Because dominant negative Ras also inhibits cyclin D1 expression and DNA synthesis on film, and the expression of these adenovirus-delivered genes does not appear until 18–24 h after infection, a role in mid to late G1 for Ras is suggested. Because Ras(V12) increases spreading, and also increases expression of cyclin D1, it is possible that spreading itself or another effector of Ras is required for the integration of MEK activity into cyclin D1 expression. Ras has a number of effectors besides Raf that can affect cell shape and cell cycle progression (67) including phosphatidylinositol 3-kinase and Raf/GDS (68), which have been demonstrated to play a role in cyclin D1 expression (48, 53, 69, 70). Thus, it is possible that phosphatidylinositol 3-kinase or Raf/GDS plays a role in allowing the cell to utilize MEK in activating cyclin D1 expression, particularly as Raf/GDS has been demonstrated to regulate cyclin D1 transcription (48).

Despite the inability of constitutively active Raf or MEK to strongly activate cyclin D1 expression on collagen gel, G1-S progression still occurred. Although cyclin D1 is often expressed in response to growth factor stimulation and is believed to be important in driving the cells into S-phase, it is not a definitive requirement for cell cycle progression, as cyclin D1 knockout mice are still viable (71). Cyclin D1 has been shown to regulate the timing of G1-S as fibroblasts lacking cyclin D1 have delayed progression into S phase, but still progress through the cell cycle (72). It is possible that the lack of cyclin D1 is compensated by other D type cyclins (72, 73) or cyclins A or E, which also act upon the Rb-E2F regulation of the cell cycle. The mechanism by which activated Raf and MEK drive progression into S phase may be related to expression of cyclin E, which appears higher in cells transfected with activated Ras pathway mutants. One role for cyclin D1/cdk4/6 is to titrate p27 or p21 from complexing with and inhibiting activity of cyclin E/cdk2 (24, 47), which we have shown to participate in the difference in cyclin D1 activity on collagen film versus denatured collagen (24). Increased expression of cyclin E may also tip the balance in favor of free cyclin E/cdk2 levels over those bound and inhibited by CKIs, negating the requirement for cyclin D1. It is also possible that activated Raf or MEK decreases expression of a CKI such as p21 or p27, allowing more active cyclin E/CDK2 complexes to form.

Together these results suggest that adhesion to polymerized collagen actively inhibits EGF signaling to cyclin D1 expression and G1-S progression through inhibition of signaling to ERK and at least one other pathway required to integrate ERK signaling into cyclin D1 expression. Determining whether the disruption in EGF signaling on collagen gel is a response to different biochemical or mechanical signals will be a future direction of this research. Understanding how cells respond to different mechanical and biochemical signals produced by the ECM will be critical for applying knowledge gained from cell culture studies to understanding how cells behave in vivo.
Collagen Regulation of ERK and Cyclin D1 in Hepatocytes

where dimensionality and mechanical properties of the matrix are vastly different from that of a rigid ECM-coated tissue culture dish.

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The Role of Collagen Structure in Mitogen Stimulation of ERK, Cyclin D1 Expression, and G1-S Progression in Rat Hepatocytes
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