Rho Kinase, Myosin-II, and p42/44 MAPK Control Extracellular Matrix-mediated Apical Bile Canalicular Lumen Morphogenesis in HepG2 Cells

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Submitted January 24, 2006; Revised April 18, 2006; Accepted April 27, 2006
Monitoring Editor: Keith Mostov

The molecular mechanisms that regulate multicellular architecture and the development of extended apical bile canicular lumens in hepatocytes are poorly understood. Here, we show that hepatic HepG2 cells cultured on glass coverslips first develop intercellular apical lumens typically formed by a pair of cells. Prolonged cell culture results in extensive organizational changes, including cell clustering, multilayering, and apical lumen morphogenesis. The latter includes the development of large acinar structures and subsequent elongated canalicular lumens that span multiple cells. These morphological changes closely resemble the early organizational pattern during development, regeneration, and neoplasia of the liver and are rapidly induced when cells are cultured on predeposited extracellular matrix (ECM). Inhibition of Rho kinase or its target myosin-II ATPase in cells cultured on glass coverslips mimics the morphogenic response to ECM. Consistently, stimulation of Rho kinase and subsequent myosin-II ATPase activity by lipoxygenase-controlled eicosatetraenoic acid metabolism inhibits ECM-mediated cell multilayering and apical lumen morphogenesis but not initial apical lumen formation. Furthermore, apical lumen remodeling but not cell multilayering requires basal p42/44 MAPK activity. Together, the data suggest a role for hepatocyte-derived ECM in the spatial organization of hepatocytes and apical lumen morphogenesis and identify Rho kinase, myosin-II, and MAPK as potentially important players in different aspects of bile canicular lumen morphogenesis.

INTRODUCTION

Hepatocytes develop from hepatoblasts and adopt a specific polarized architecture and therewith correlated functions in response to proper environmental cues (reviewed in Kinoshita and Miyajima, 2002; Lemaigre and Zaret, 2004; Zhao and Duncan, 2005). In vivo, fetal hepatocytes develop in cords that are three to five cells thick, which are reduced to one- or two-cell–thick cords in mature hepatocytes. In the cords, each hepatocyte is attached to its neighbors in a two-dimensional plate. On either side of the cord, each hepatocyte faces the space of Disse, across which it communicates freely with adjacent blood-filled sinusoids. In contrast to simple epithelial cells, which sit on their basal surface and have an apical surface exposed to the external space while attached to their neighbors along the lateral surface, hepatocytes display a unique topology (Figure 1). Thus, a typical hepatocyte displays two basal surfaces on opposite ends of the cell where it faces the sinusoids on either side of the cord in which it resides (reviewed in Stamatoglou and Hughes, 1994). A portion of the lateral domain of hepatocytes is modified to form the apical surfaces that line the bile canaliculi, which in vivo form an intercellular network of narrow passages contained within each cord. The bile canaliculi are thought to develop from small vacuoles between adjacent hepatocytes before bile formation starts, consistent with the existence of functional mechanisms for sorting of plasma membrane molecules early in development (Feracci et al., 1987). In the primordial stage, the small vacuoles give rise to extended bile canaliculi that line several hepatocytes. Functional bile canicular lumens are separated from the perihepatocytic space by the presence of tight junctions and desmosomes.

An early step in the development of canalicular lumens is the biogenesis of apical plasma membranes to accommodate bile canicular functions. Studies with primary hepatocyte cultures and hepatic cell lines have revealed several processes that contribute to apical plasma membrane biogenesis and the development of intercellular apical vacuoles (reviewed in Zegers and Hoekstra, 1997; Gallin, 1997; Wang and Boyer, 2004). Signaling cascades induced by interleukin-6 family cytokines such as oncostatin M stimulate fetal...
liver development, establishment of intercellular contacts, and the formation of apical plasma membranes (Matsui et al., 2002; van der Wouden et al., 2002). Oncostatin M-stimulated apical plasma membrane biogenesis requires protein kinase A, which has been implicated in apical surface-directed membrane trafficking (van IJzendoorn et al., 1997; Zegers and Hoekstra, 1998; Roelofsen et al., 1998; van IJzendoorn and Hoekstra, 1998), and, in this way, regulate the supply of specific proteins and lipids from subapical recycling endosomes to the developing apical surface (reviewed in van IJzendoorn and Hoekstra, 1999; Hoekstra et al., 2004). Furthermore, the regulated exchange of proteins and lipids between the endosomal system and developing apical surface is tightly controlled by dihydroceramide synthase-regulated sphinganine metabolism (van IJzendoorn et al., 2004b); the cell cycle regulatory proteins p27Kip1 and cyclin-dependent kinase 2 (van IJzendoorn et al., 2004a); and small GTPase rab proteins, including rab11 (Wakabayashi et al., 2005) and rab3d (Larkin et al., 2000). Although these and other studies have revealed some of the molecular mechanisms that control the biogenesis of apical plasma membrane domains in hepatocytes, and, in this way, the early steps in the formation of intercellular vacuoles with compositional and functional characteristics of bile canaliculi, polarizing hepatic cell lines (e.g., HepG2 or WIF-B; Ihrke et al., 1993), and primary hepatocytes in culture do not typically form subsequent elongated canalicular networks as observed in vivo. Consequently, virtually nothing is known about the molecular mechanisms that control the formation of multilobar apical canaliculi.

The spatial organization and polarized architecture of hepatocytes is likely to be dictated by interaction of the cells with the extracellular environment, e.g., neighboring cells and the extracellular matrix (ECM; reviewed in Stamatoglou and Hughes, 1994). The space of Disse contains most major ECM molecules, the majority of which are derived from endothelial cells and hepatic stellate (Ito) cells (Friedman et al., 1985; Senoo et al., 1998). In addition, hepatocytes produce ECM (Selden et al., 2000), albeit at significantly lower quantities. Some isolated ECM components have been reported to induce transcriptions of a subset of liver-specific genes (DiPersio et al., 1991) and to facilitate the formation of extended apical canaliculi in hepatocytes cultured either in suspension (Tzanakakis et al., 2001; Ábu-Abasi et al., 2002) or in an ECM “sandwich” configuration (Berthiaume et al., 1996; Moghe et al., 1996). It is not clear, however, whether hepatocyte-derived ECM contributes to hepatocytic morphogenetic processes, and, importantly, which molecular mechanisms and signaling pathways are involved in bile canalicular lumen morphogenesis.

The aim of this study was to find culture conditions and molecular parameters that promote the development of multicellular canalicular lumens. It is demonstrated that human hepatoma HepG2 cells cultured on coverslips first develop intercellular apical vacuoles between two or three neighboring cells. On the deposition of HepG2 cell-derived ECM molecules, however, the cells display clustering, multilayering, and a dramatic remodeling of the apical vacuoles resulting in the development of extended apical lumens that span multiple cells, and resemble the organizational pattern during development, regeneration, and neoplasia of the liver. Our data indicate a critical role for Rho kinase and myosin-II ATPase activity as well as a requirement for p42/44 MAPK signaling in cellular reorganization and/or apical lumen morphogenesis mediated by hepatocyte-derived ECM.

**MATERIALS AND METHODS**

DMEM and Hank’s balanced salt solution (HBSS) were from Invitrogen (Carlsbad, CA). Y-27632 was obtained from Calbiochem (San Diego, CA). Eicosa-SZ, S/Z,11,14-tetraenoic acid (eicosatetraenoic acid; ETA), nordihydroguaiaretic acid (NDGA), and 2,3-dinitrobenzene monoxide (BOM) were purchased from Sigma-Aldrich (St. Louis, MO). Blebbistatin was obtained from Toronto Research Chemicals (North York, Ontario, Canada). 2′-Amino-3′-methoxyflavone (PD98059) was purchased from LC Laboratories (Woburn, MA). Mouse monoclonal anti-villin and monoclonal anti-ZO-1 antibodies were from Transduction Laboratories (Newinton, NH) and Zymed Laboratories (South San Francisco, CA), respectively. Mouse monoclonal antibodies (mAbs) against MLC2, phospho (ser19)-MLC2, p42/44 MAPK, and phospho-p42/p44 MAPK were from Cell Signaling Technology (Beverly, MA). A mouse mAb against MRP2 (M2 III-6) was obtained from Abcam (Cambridge, MA), and a polyclonal antibody raised against fibronectin was purchased from Chemicon International (Temecula, CA). Alexa-488- or Alexa-596-conjugated secondary antibodies and 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminohexanoic acid (C6-AMCA) were obtained from Molecular Probes (Eugene, OR).

**Inhibitors**

Pharmacological inhibitors were used at concentrations and time intervals reported previously in the literature and/or indicated by the manufacturer: Y-27632, 2.5 μM for 24–72 h, which effectively inhibits phosphorylation of myosin-II by Rho kinase in response to ETA (cf. Figure 7); ETA, 50 μM for 24–72 h, previously shown to stimulate Rho kinase (Araki et al., 2001); NDGA, 10 μM, which effectively blocks ETA-induced effects in HepG2 cells (cf. Figure 6); and BDM and blebbistatin, 10 μM and 100 μM, respectively, for 24 h. In control experiments, cells were treated with vehicle only.

**Cell Culture**

Human HepG2 cells (ATCC HB0065) were cultured in DMEM containing 4500 mg of glucose/1 supplemented with 10% heat-inactivated (30 min at 56°C) fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (5% CO2 in air) at 37°C. Media were changed every other day. Cells were grown in 25-cm² culture flasks or plated at low density onto ethanol-sterilized glass coverslips for experiments.

**ECM Deposition**

HepG2 cells were plated on coverslips and cultured until a confluent layer was formed (typically 3–5 d). To remove the cells without affecting the deposited ECM, use of digestive proteases such as trypsin was avoided. Instead, the cells were incubated with 2 ml of distilled water per coverslip for 45 min at 37°C. Cells were removed from the coverslips by thorough resuspension. The coverslips were then carefully examined under a light microscope to verify that the cells and cellular debris had been removed. The effective removal of cells and cellular debris with this method was also verified by scanning electron microscopy and (immuno)fluorescent labeling of the decellularized coverslips with the DNA stain 4,6-diamidino-2-phenylindole (DAPI) or antibodies against actin and tubulin. No cells or cellular debris (Supplemental Figure S1D versus S1A; S1H versus S1G), DNA (Supplemental Figure S1E versus S1B), or cytoskeleton remnants (our unpublished data) were present on the coverslip after removal of the cells. Importantly, using antibodies against fibronectin (diluted 1:80), it was demonstrated...
that ECM remains on the coverslip after removal of the cells (Supplemental Figure S1I). Fibillar ECM-like structures could occasionally be observed on decellularized coverslips with scanning electron microscopy (Supplemental Figure S1I and S1J). Only coverslips on which cellular remnants could not be detected with light microscopy were used, and they were subsequently incubated with DMEM for 1 h at 37°C to enable reformation of matrix proteins. HepG2 cells were plated at low density on the ECM for 24, 48, or 72 h.

**Determination of Cell Polarity**

Cells were fixed in −20°C absolute ethanol for 10 s and rehydrated in HBSS. Cells were double stained with a mixture of the nuclear stain Hoechst-33528 (5 ng/ml) and tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (100 ng/ml) in HBSS for 20 min at room temperature (RT). After washing, coverslips were blocked with 1% BSA (wt/vol in HBSS; pH 7.4) for 30 min at RT and then incubated with a mouse mAb against the apical protein villin conjugated with Alexa Fluor-488 (Molecular Probes) for 45 min at RT. After staining for the tight junction protein ZO-1, MRP2, or (phospho-)MLC2, cells were washed three times with cold HBSS and mounted. Cells were double stained with a mixture of the nuclear stain Hoechst-33528 (1:100 dilution in HBSS) for 2, 1, and 1 h at RT, respectively. Cells were washed three times with HBSS and mounted. To verify that apical lumens were enclosed within cell clusters, serial confocal x-y sections (typically 1 section/0.5 μm) and subsequent x-z reconstructions were prepared using an acousto-optical beam splitter-based confocal microscope (Leica Microsystems, Heidelberg, Germany). Animated serial x-y sections and corresponding projections were exported as AVI-formatted movie clips using Leica confocal software Lite version 2.61.

**Immunostaining**

For villin staining, cells were fixed and permeabilized with −20°C acetone for 5 min. After washing with HBSS, cells were blocked in HBSS containing 1% bovine serum albumin (BSA) (wt/vol in HBSS; pH 7.2) for 30 min at RT and subsequently incubated with a mouse mAb against the apical protein villin (1:50 dilution in HBSS) for 2 h at RT. Cells were washed to remove primary antibody, followed by incubation with the secondary goat anti-mouse antibody (2 μg/ml) conjugated with Alexa Fluor-488 (Molecular Probes) for 45 min at RT. For staining for the tight junction protein ZO-1, MR2, or (phospho)-MLC2, cells were fixed in 4% paraformaldehyde (wt/vol in HBSS; pH 7.2). After washing (5 times in HBSS), cells were permeabilized with 0.1% Triton X-100 (wt/vol in HBSS) for 5 min at RT. Cells were washed and blocked with 1% BSA (wt/vol in HBSS; pH 7.4) for 30 min at RT. BSA was removed, and cells were incubated with the mouse mAb anti-ZO-1 (1:100 dilution in HBSS), anti-MRP2 (1:200 dilution in HBSS), or (phospho)-MLC2 (1:100 dilution in HBSS) for 2, 1, and 1 h at RT, respectively. Cells were washed three times with HBSS and incubated with the secondary antibodies (2 μg/ml) conjugated with Alexa Fluor-488 or -596 (diluted 1:400 in HBSS; Molecular Probes) for 45 min at RT. After incubation with the secondary antibody, cells were washed three times with HBSS and mounted.

**Membrane Labeling with Fluorescent Lipid**

C2-NBD-sphingomyelin (SM) was dried under N2, and redissolved in absolute ethanol. The lipid was used at a concentration of 4 μM in HBSS [final ethanol concentration 15% (vol/vol)]. Cells were washed three times with cold HBSS and incubated with C2-NBD-SM for 30 min at 4°C. To remove noninternalized probes, cells were washed three times with 4°C HBSS, and further incubated with HBSS for 30 min at 37°C. After a subsequent three washes with 4°C HBSS, cells were incubated with BSA (5% [wt/vol]; pH 7.4) in HBSS, two times for 30 min at 37°C (back exchange). Cells were washed three times and examined immediately using an epifluorescence microscope (Provis AX70; Olympus).

**Rhodamine 123 (R123) Incubation**

Cells were washed three times with 37°C HBSS and incubated with R123 for 30 min at 37°C. R123 was added to the apical membrane and pumped into the BC by ATP-binding cassette transporters. After a wash with 37°C HBSS, cells were examined immediately.

**Multilayering**

Multilayering was expressed as the percentage of total number of cells (identifiable by fluorescently labeled nuclei) that multilayer calculated by the following equation: multilayering = (nuclei_{multilayer} – nuclei_{single})/nuclei_{total} × 100%, in which nuclei_{single} refers to nuclei that do not visibly overlap with other nuclei in the x-z direction. At least 10 fields per coverslips, each containing >50 cells, were analyzed.

**Western Blotting**

Cells were lyzed in lysis buffer (10 mM triethanolamine, pH 7.4, 1% Triton X-100, 0.1% SDS, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3P04, and 2 mM sodium vanadate, and a cocktail of protease inhibitors). Lysates were boiled for 5 min and cleared by centrifugation. Protein concentrations were determined by a bichinonic acid protein assay (Sigma-Aldrich), and equal amounts of proteins were separated on 10% SDS-PAGE gels, immunoblotted with (phospho)-MLC2 or (phospho)-p42/44 MAPK antibodies and detected with an enhanced chemiluminescence system (Amersham, Piscataway, NJ). Bands representing (phospho)-MLC2 were quantified using Scion Image software.

**Electron Microscopy**

Transmission Electron Microscopy (TEM). Cells were washed several times with 6.8% saccharose to remove serum in 0.1 M cacodylate buffer, pH 7.4, at RT and then fixed for 30 min at RT in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The cells were rinsed three times in the same buffer with 6.8% sucrose and subsequently postfixed in 2% OsO4/3% K2Fe(CN)6 in 0.2 M cacodylate buffer, pH 7.4, at 4°C for 1 h. After rinsing in 0.1 M cacodylate buffer, pH 7.4, and dehydrogenation in a graded alcohol series, the cells were embedded in Epon 812 and polymerized for 2 d at 58°C. Finally, ultrathin sections (60 nm) were cut and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM 100 electron microscope operating at 80 kV, and micrographs were taken.

Scanning Electron Microscopy. Coverslips with cells or after cell removal were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and washed (0.2 M cacodylate buffer, pH 7.4, for 15 min and subsequently with distilled water for 10 min). Samples were then dehydrated with ethanol (30, 50, 70, 96, and 100% in steps of 10 min, followed by two times 30 min with 100% ethanol). Samples were subsequently dried with a Baltec CPD 100 critical point dryer, allowing ethanol to CO2 exchange. Sample coating was done with Au/Pd (~3 nm (Sputtercoater Balzers 120B), and samples were analyzed using a JEOL JSM-6301F cold field emission scanning electron microscope operating at 2 kV and at 60 or 600× magnification.

**Statistics**

A two-tailed unpaired Student’s t test (assuming equal variances) was used to assess whether the means of two data sets were statistically different. The assumption of normality for the performed t tests was verified with a Kolmogorov–Smirnov test.

**RESULTS**

**Apical Lumen Formation in HepG2 Cells**

Human hepatoma HepG2 cells (Knowles et al., 1980) provide a cell culture model system to study modulation of the liver phenotype that occurs during fetal/adult development or during liver regeneration (Kelly and Darlington, 1980), and they have been shown to be an excellent model for the study of apical plasma membrane biogenesis, i.e., cell polarity development (Bouma et al., 1989; Chiu et al., 1990; Sormunen et al., 1993; Zaal et al., 1994; Zegers et al., 1998; van IJzendoorn and Hoekstra, 1999). When cultured on glass coverslips, HepG2 cells acquire a polarized phenotype, demonstrated by the appearance of apical lumens (BC) between adjoining cells, in a time-dependent manner (Figure 2A). BC are readily visualized by the presence of apical resident proteins (Roelfsen et al., 2000; Ait Slimane et al., 2003) and by TRITC-phalloidin staining of the F-actin cytoskeleton, which is abundantly present underneath the apical surface (Figure 2D; Zegers and Hoekstra, 1998). Determination of the ratio BC/100 cells (a measure for HepG2 cell polarity development; see Materials and Methods; cf. van IJzendoorn and Hoekstra, 1997, 1998; Zegers and Hoekstra, 1997) at 24, 48, and 72 h after plating reveals an initial steady increase in the number of apical lumens in time and a subsequent decrease after 48 h (Figure 2A). Approximately 25 BC/100 cells were counted in 48-h-old cell cultures, which decreased to 20 BC/100 cells in 72-h-old cell cultures (Figure 2A; cf. van IJzendoorn and Hoekstra, 1997, 1998). A representative image of a 72-h-old polarized HepG2 cell culture, showing TRITC-phalloidin–labeled BC and Hoechest-33528–stained nuclei, is depicted in Figure 2, C–E.

We next determined the number of cells that participated in a single BC as a function of time. For this, cells were fixed at the different time points and stained with TRITC-phalloidin and Hoechest-33528. TRITC-phalloidin–labeled BC were
identified, and the number of cells associated with the BC was determined. Interestingly, the number of cells sharing a single BC (ratio cells/BC) increased in time. Thus, whereas 24 h after plating 80% of the BC were shared by two cells (couplets), 48–72 h after plating this percentage was reduced to 50–60%, whereas other BC were shared by three (>30% of all BC) or even more than five (~15% of all BC) cells (Figure 2B, Ctrl; p < 0.05). These data suggest that, in addition to the previously reported formation of apical lumens between two adjacent cells, subsequent apical lumen morphogenesis occurs in time to form larger lumens that span multiple cells.

**HepG2 Cells Plated on Predeposited ECM Display Clustering, Cell Multilayering, and Remodeling of Apical Lumens**

Because the number of HepG2 cells participating in a single apical lumen increased as a function of time (Figure 2B), which would be consistent with the deposition of increasing amounts of ECM by the cells, we next investigated the effect of ECM on apical lumen formation in HepG2 cells by culturing the cells on predeposited HepG2 ECM. To obtain coverslips coated with HepG2 ECM, HepG2 cells were first grown onto sterilized glass coverslips until confluence (3–5 d). Then, the cells were removed from the coverslips with distilled water, leaving deposited ECM on the coverslip (see Materials and Methods and Supplemental Figure S1). The ECM-coated coverslips were subsequently incubated with DMEM at 37°C and used for the culture of a new batch of cells. After different times of culturing, cells grown on predeposited ECM were fixed and double stained with TRITC-labeled phalloidin to visualize the apical lumens and Hoechst-33528 to visualize the nuclei. As shown in Figure 2, F–H, HepG2 cells displayed a dramatically altered spatial organization when cultured on deposited ECM. Compared to cells grown on glass coverslips (Figure 2, C–E), deposited ECM (F–H), Hoechst-stained nucleus in C and F, TRITC-phalloidin stained BC in D and G, and merged pictures in E and H. Bars, 10 μm. (I and J) TEM image of polarized HepG2 cells plated on glass coverslips (I) or predeposited ECM (J). N, nucleus. Bar, 5 μm.

![Figure 2. ECM affects apical lumen morphology in HepG2 cell cultures. (A) Apical polarity development in HepG2 cells as a function of time after plating, expressed as the ratio BC/100 cells. Data are expressed as mean ± SD of three independent experiments carried out in duplicate. (B) Average number of cells participating in a single BC (expressed as the ratio cells/BC) as a function of time after plating on glass coverslips (Ctrl) or predeposited ECM (ECM). Data are expressed as mean ± SD of three independent experiments carried out in duplicate. White, hatched, gray, and dotted bars represent BC shared by two, three, four, or more than five cells, respectively. Student’s t test to determine the statistical significance between Ctrl and ECM at 24 h: white bars, p < 0.02; hatched bars, p < 0.003; gray bars, p = 0.11; dotted bars, p = 0.2; at 48 h: white bars, p < 0.003; hatched bars, p = 0.4; gray bars, p < 0.01; dotted bars, p < 0.05; at 72 h: white bars, p < 0.01; hatched bars, p = 0.26; gray bars, p = 0.07; dotted bars, p < 0.05. Student’s t test to determine the statistical significance of control cells between 24 and 48 h, p < 0.05; between 48 and 72 h, p < 0.01. (C–H) Polarized morphology of HepG2 cells plated on glass coverslips (C–E) or predeposited ECM (F–H). Hoechst-stained nucleus in C and F, TRITC-phalloidin stained BC in D and G, and merged pictures in E and H. Bars, 10 μm. (I and J) TEM image of polarized HepG2 cells plated on glass coverslips (I) or predeposited ECM (J). N, nucleus. Bar, 5 μm.

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do not overlap in the x-z direction with other nuclei (see Materials and Methods). At least 10 fields per coverslips, each containing >50 cells, were analyzed. Whereas only 10 ± 3% of the cells cultured on glass coverslips displayed multilayering, 47 ± 6% of the cells cultured on predeposited ECM multilayered (p value from an unpaired t test = 0.03).

Most strikingly, culturing the cells on predeposited ECM induced the formation of large elongated apical lumens that span multiple cells, resembling the first clear indication of parenchymal organization in embryonic and regenerating liver (Ogawa et al., 1979; Stamatoglou et al., 1992). Indeed, whereas in control cells BC are typically located between two neighboring cells, in cells cultured on ECM, BC were predominantly shared by multiple, sometimes up to 10 cells (Figure 2, F–H, compare with C–E). The association of multiple cells with a single lumen in cells cultured on predeposited ECM was confirmed by TEM (Figure 2, I and J). When HepG2 cells were cultured on coverslips coated with only laminin, collagens, fibronectin, or the integrin nonspecific substrate poly-l-lysine, remodeling of apical lumens was not observed (our unpublished data). Furthermore, culturing the cells in low-serum (0.5% FCS) or serum-free medium did not change the outcome of the experiments (our unpublished data), suggesting that serum-derived factors may not be critical for ECM-mediated cell reorganization and apical lumen remodeling.

To add a quantitative measure to the ECM-mediated apical lumen remodeling, the number of cells that shared a single BC was determined as a function of time in cell cultures grown on glass coverslips or predeposited ECM. In Figure 2B, the percentage of BC shared by two cells (white bars), three cells (hatched bars), four cells (gray bars), or more than five cells (dotted bars) in cell cultures grown on glass coverslips (Ctrl) and predeposited ECM (ECM) is shown. Whereas in cultures plated on glass coverslips the percentage of BC shared by two cells was >80% at 24 h and gradually decreased to 50–60% at 72 h, in cultures grown on predeposited ECM for 24 h the percentage of BC shared by two cells was dramatically reduced to ~50% and continued to decrease to ~40% at 72 h. Most strikingly is the approximately twofold increase (from 15 to 30%; p < 0.05) in the percentage of BC shared by four or more than five cells in cultures grown for 72 h on predeposited ECM, compared with cells grown on glass coverslips (Figure 2B). Together, these data suggest that hepatocytes can modulate their own cell-to-cell organization and polarized morphology through the deposition of ECM.

**ECM-mediated Apical Lumen Remodeling Does Not Perturb Apical Plasma Membrane Characteristics and Functioning**

We next determined whether the membranes lining the remodeled apical lumens in ECM-grown cell cultures displayed typical apical plasma membrane characteristics. First, we verified that apical resident proteins such as MDR1 (Ait Slimane et al., 2003) and villin were exclusively localized to the BC (Figures 3, A–C. and 4, C and D, respectively). Moreover, apical plasma membrane-associated MDR1 readily translocated R123, a fluorescent substrate for MDR1 remaining at the basolateral domain was observed, indicating a proper fence function of the tight junctions. Together, these data suggest that cells cultured on predeposited ECM for 72 h displayed typical characteristics and functioning of the canalicular plasma membrane.

**Inhibition of Rho Kinase (ROCK) Mimics ECM-mediated Cell Reorganization and Apical Lumen Remodeling**

The small GTPase Rho and its downstream effector Rho kinase (ROCK) have recently been implicated in the regulation of polarized epithelial cell architecture (Sahai and Marshall, 2002; Vaezi et al., 2002; Omelchenko et al., 2003; Wozniak et al., 2003; Eisen et al., 2004; Prahalad et al., 2004), presumably by interpreting extracellular signals such as those provided by the ECM. To investigate the possible involvement of ROCK in the ECM-mediated HepG2 cell remodeling, cells were first cultured on glass coverslips in the absence or presence of the specific ROCK inhibitor Y-27632 (2.5 μM). In the absence of the ROCK inhibitor, HepG2 cells formed a monolayer and developed BC in between two or three cells (Figure 4, A and B; cf. Figure 2, C–E). In striking contrast, inhibition of ROCK induced clustering and multilayering (>10-fold compared with nontreated cells) of the cells and induced remodeling of the apical lumens, similarly as observed in cells cultured on predeposited ECM (Figure 4, C and D; compare with Figure 2, F–H). Determination of the percentage of BC shared by two, three, four, or more than five cells showed a more than twofold increase in the number of BC shared by four or more than five cells (Figure 4E, gray and dotted bars, respectively), whereas the percentage of BC shared by two or three cells (Figure 4E, white and hatched bars) was reduced from >80% in control cells to ~50% in Y-27632-treated cells. The large apical lumens in ROCK-inhibited cells were lined with a dense F-actin network (Figure 4, K and M) and contained the microvilli protein villin (Figure 4D). The integrity of the apical plasma membrane was unperturbed, verified similarly as described for cells cultured on predeposited ECM (our unpublished data; cf. Figure 3 and Supplimentary Figure S2).
Multicellular apical lumens may form de novo and/or develop at the expense of existing individual BC. To investigate this, we cultured cells on glass coverslips for 2 d in normal cell culture medium, and, subsequently, for another 24, 48, or 72 h in the presence of Y-27632. After 2 d in culture, apical lumens were predominantly observed between two or three cells (Figure 4, F and G, arrows; cf. Figure 2, C–E). After a subsequent 24-h culture in the presence of Y-27632, cells had clustered and in the cell clusters multiple larger apical lumens could be observed (Figure 4, H and I). After another 24 h in the presence of Y-27632, cell multilayering became apparent and larger multicellular apical lumens were typically observed in cell clusters, often with other smaller apical lumens present in the same cell cluster (Figure 4, J and K, arrows). The smaller apical lumens disappeared in time, leaving a single large and often elongated multicellular apical lumen (Figure 4, L and M). Along with this apparent transition of small lumens in between 2 cells to larger multicellular lumens, the absolute number of apical lumens in Y-27632-treated cell cultures decreased in time (Figure 4N). Not only Y-27632-treated cells but also non-treated cells plated on glass coverslips for up to 96 h continued to develop larger multicellular lumens (>4 or 5 cells/lumen; p < 0.05) with a concomitant decrease in the number of small lumens in between two or three cells (Figure 5A; p < 0.05), and with a concomitant decrease in the total number of lumens in the culture (Figure 5B). Moreover, laser scanning confocal microscopy and subsequent three-dimensional reconstruction of apical lumens in HepG2 cells cultured on predeposited ECM for 3 d (D, phase contrast; E, R123; and F, merged image). (G–I) Tight junction protein ZO-1 (H) borders the apical domain, visualized with TRITC-phalloidin (G). Merged image in I. (J and K) TEM image of the apical lumen (J, black asterisk) and apical junctions (K, arrows) of cells (indicated with white asterisks in J) cultured on predeposited ECM. (L) C6-NBD-SM at the basolateral surface does not diffuse to the apical membrane (asterisk) of cells cultured on predeposited ECM. (M) C6-NBD-SM in the apical plasma membrane domain of cells cultured on predeposited ECM does not diffuse to the basolateral domain. Bars in A–L, L, and M, 10 μm. Bars in J and K, 2 μm and 100 nm, respectively.

ETA Metabolism-stimulated ROCK Activity Prevents ECM-mediated Cell Reorganization and Apical Lumen Remodeling

The virtually identical morphological effects of Y-27632 compared with the effects of predeposited ECM strongly suggest that inhibition of ROCK represents a key factor in the ECM-mediated cell reorganization and apical lumen remodeling. To investigate this, we treated cells cultured on predeposited ECM with ETA (50 μM), known to stimulate Rho signaling and ROCK activity (Feng et al., 1999; Araki et al., 2001). Indeed, the formation of stress fibers at the basal...
surface of HepG2 cells (our unpublished data), and a Y-27632–sensitive increase in the phosphorylation status of the well-known ROCK target myosin light chain-2 (MLC2) at serine-19 (Totsukawa et al., 2000; Figure 7A), confirmed that ETA stimulates ROCK activity. The addition of ETA in the cell culture medium completely blocked the ECM-mediated cell reorganization and apical lumen remodeling (Figure 6B, compare with A). Thus, whereas cell reorganization and apical lumen remodeling was apparent in cultures grown on predeposited ECM (Figure 6A), treatment with ETA resulted in monolayer cell cultures with small apical lumens in between two or three cells (Figure 6B), indistinguishable from control cells grown on glass coverslips (cf. Figure 2, C–E).

Indeed, determination of the percentage of BC shared by two cells (white bars), three cells (hatched bars), four cells (gray bars), or more than five cells (dotted bars) in ETA-treated cultures showed a complete block of the effects of predeposited ECM and no significant differences compared with cells grown on glass coverslips (Figure 6E). Interestingly, the inhibitory effect of ETA was rescued when cells were cotreated with the lipoxygenase inhibitor NDGA (10 μM; Figure 6, C and E), which blocks the conversion of ETA to leukotrienes, the latter of which have been reported to mediate the ETA-stimulated Rho signaling (Setoguchi et al., 2001; Thodeti et al., 2002; Roberts et al., 2003). Together, these data strongly suggest that ECM induces cell reorganization and apical lumen remodeling by down-regulating ROCK activity. In addition, lipoxygenase-controlled ETA metabolism antagonizes ECM-mediated cell reorganization. Importantly, treatment of the cells with ETA did not inhibit apical lumen formation per se (Figure 6B), suggesting that the development of apical lumens between adjacent cells and the subsequent remodeling of apical lumens to form multicellular canals are controlled by distinct signaling pathways.

We next investigated whether cell clustering, multilayering, and apical lumen remodeling required a continuous down-regulation of ROCK activity. For this, cells were cultured on predeposited HepG2 ECM for 72 h to allow the morphological response as described in Figures 2 and 3, after which the cells were treated for another 24 h in the presence of ETA. As shown in Figure 6D, the ECM-mediated morphological changes could not be reversed by ETA. However, it was readily observed that the apical lumens displayed a more constricted morphology compared with cells that had not been treated with ETA (Figure 6D; cf. A). These data suggest that the ECM-mediated and ROCK down-regulation-mediated effects on cell clustering, multilayering, and apical lumen remodeling do not require continuous down-regulation of ROCK activity, and, in addition, suggest that ROCK activity is actively involved in regulating contraction and shape of the multicellular apical lumens.
Inhibition of Myosin II ATPase Mimics the Effects of ECM or ROCK Inhibition on Cell Reorganization and Apical Lumen Remodeling

Membrane contraction, including the bile canalicular membrane (Tsukada et al., 1995), is typically controlled by actin–myosin interactions. MLC2 is a well-characterized downstream target of ROCK, and its phosphorylation at serine-19 results in increased actin–myosin-based contractility. In polarized HepG2 cells, MLC2 predominantly localizes to the apical domain of the cells (Figure 7C), revealing a pattern reminiscent of that observed for tight junction proteins (cf. van der Wouden et al., 2002). Phosphorylated MLC2 also

Figure 5. Apical lumen remodeling in cells cultured on glass coverslips for >48 h. Cells were cultured on glass coverslips for 48 h and subsequently for another 0, 24, or 48 h. Cells were then fixed, and the ratio cells/BC (A) and BC/100 cells (B) was determined as described in Materials and Methods. Data are expressed as mean ± SD of three independent experiments carried out in duplicate. *p < 0.05. (C–E) Cells cultured on glass coverslips for 72 h were fixed and (immuno)labeled with anti-MRP2 antibodies (C–C') and TRITC-labeled phalloidin (D) and subjected to laser scanning confocal microscopical. Multiple x-y sections (1/0.5 μm) were superimposed. A merged image is shown in E. C' and C'' are enlargements of the respective boxes in C, and the numbers indicate distinct apical lumens.

Figure 6. Effects of ETA metabolism on ECM-mediated apical lumen remodeling. (A) TRITC-phalloidin-labeled apical lumen in cells cultured on ECM for 3 d. (B) TRITC-phalloidin–labeled apical lumen in cells cultured on ECM for 3 d in the presence of ETA. (C) TRITC-phalloidin–labeled apical lumen in cells cultured on ECM for 3 d in the presence of ETA + NDGA. (D) TRITC-phalloidin–labeled apical lumen in cells cultured on ECM for 48 h, followed by treatment with Y-27632 for 24 h. Bar, 10 μm. (E) Average number of cells that participate in a single BC of HepG2 cells cultured on glass coverslips (Ctrl), ECM, ECM + ETA, or ECM + ETA + NDGA for 24 h. White, hatched, gray and dotted bars represent BC shared by two, three, four, or more than five cells, respectively. Student’s t tests: white bars, Ctrl versus ECM, p < 0.001; ECM versus ECM + ETA, p = 0.001; ECM versus ECM + ETA + NDGA, p < 0.001; hatched bars, Ctrl versus ECM, p = 0.006; ECM versus ECM + ETA, p = 0.44; ECM + ETA versus ECM + ETA + NDGA, p = 0.42; gray bars, Ctrl versus ECM, p = 0.90; ECM versus ECM + ETA, p = 0.14; ECM + ETA versus ECM + ETA + NDGA, p = 0.03; and dotted bars, Ctrl versus ECM, p = 0.008; ECM versus ECM + ETA, p = 0.014; ECM + ETA versus ECM + ETA + NDGA, p = 0.39.
localizes to the apical domain, and, in addition, was observed at the nucleus (Figure 7D). Treatment of the cells with 50 μM ETA, which inhibits ECM-mediated apical lumen remodeling, increases MLC2 phosphorylation in a Y-27632-sensitive manner (Figure 7, A and B), whereas culturing HepG2 cells on predeposited ECM results in a reduced level of phosphorylated MLC2 (Figure 7, A and B). These data suggest that the effects of ECM and ROCK inhibition on cellular reorganization and apical lumen remodeling may be due to a decrease in myosin function. To further investigate this, we examined whether inhibition of myosin ATPase activity could mimic the effects of ECM and ROCK inhibition. For this, cells were cultured on glass coverslips in the absence or presence of the myosin heavy chain ATPase inhibitor BDM (10 mM). As shown in Figure 7F, cells treated in the presence of BDM displayed similar cell clustering, multilayering, and apical lumen morphogenesis as observed in cells cultured on predeposited ECM (cf. Figure 2, F–H) or in the presence of 2.5 μM Y-27632 (cf. Figure 4, A–D). Determination of the percentage of BC shared by two, three, four, or more than five cells showed a more than 10-fold increase (p = 0.001) in the number of BC shared by four or more than five cells (Figure 7G, gray and dotted bars, respectively), whereas the percentage of BC shared by two or three cells (Figure 7G, white and hatched bars, respectively) was reduced from >80% in control cells to ~45% in Y-27632-treated cells (p < 0.001). The effects of BDM could not be counteracted by the addition of ETA to the culture medium (our unpublished data), suggesting that myosin-II ATPase acts downstream of ROCK. The effects of BDM on cell clustering and apical lumen remodeling could be reproduced with 100 μM blebbistatin, another inhibitor of myosin-II ATPase (Supplemental Figure S3). Together, these data strongly suggest that the effects of predeposited ECM and ROCK inhibition are, at least in part, mediated by the reduced phosphorylation status of MLC2 and consequent inhibition of myosin-II heavy chain ATPase activity.

Apical Lumen Morphogenesis but Not Cell Multilayering Requires p42/44 MAPK

MAPKs (p42/44 MAPK) can be activated upon extracellular cues and have been implicated in epithelial multilayering and apical morphogenesis (O’Brien et al., 2004). To examine the role of p42/44 MAPK in the ECM-mediated cell clustering, multilayering, and apical lumen remodeling, cells were cultured on predeposited ECM for 72 h in the presence of 50 μM PD98059, a selective inhibitor of the mitogen-activated protein kinase kinase (MEK)-p42/44 MAPK signaling pathway. In the presence of PD98059, HepG2 cells cultured on predeposited ECM failed to undergo apical lumen remodeling (Figure 8, A–C) as observed in nontreated cells (Figure 8, D–F). Indeed, the ratio cells/BC in cells cultured on predeposited ECM in the presence of PD98059 was similar to that observed in control cultures grown on glass coverslips (Figure 8G). However, PD98059-treated cells did cluster and displayed cell multilayering on ECM to a similar extent compared with nontreated cells (Figure 8G). Similar data were obtained with another inhibitor of the MEK–MAPK signaling pathway, UO126 (10 μM; our unpublished data). These data suggest that p42/44 MAPK activity is required at a stage subsequent to cell multilayering but before apical lumen remodeling. We did not observe an alteration in the level of phosphorylated p42/44 MAPK in cells cultured on predeposited ECM or in the presence of Y-27632 (Figure 8I), suggesting that endogenous MAPK signaling may act as a dominant parallel signaling pathway.
resemble the first clear indication of parenchymal organization (Figure 3). The multicellular apical lumens closely resemble the first clear indication of parenchymal organization (Figure 3). The multicellular apical lumens closely separated from the basolateral space by adherens and tight junctions (Figure 3). The multicellular apical lumens closely resemble the first clear indication of parenchymal organization (Figure 3). The multicellular apical lumens closely separated from the basolateral space by adherens and tight junctions (Figure 3).

DISCUSSION

The molecular mechanisms and signaling cascades that contribute to the development and morphogenesis of apical lumens in hepatocytes and other epithelial cells are not well understood, but are clearly regulated by the extracellular environment, including adjacent cells and the ECM (Ojakian et al., 1997; Yeaman et al., 1999). When plated on glass coverslips, hepatoma HepG2 cells grow as a monolayer culture, and, in time, develop apical vacuoles in between two or three cells (Figure 2), consistent with previous reports (Bouma et al., 1989; Chiu et al., 1990; Sormunen et al., 1993; van IJzendoorn et al., 1997; Falasca et al., 1999; Ishigami et al., 2005). When plated on coverslips onto which ECM had previously been deposited by HepG2 cells, the spatial organization of the cells dramatically and rapidly changes, evidenced by the self-assembly of cells in multilayering cell clusters. In addition, apical lumens readily form larger, elongated structures that span multiple cells (Figures 2 and 3). The membranes delineating these multicellular lumens display proper apical bile canicular plasma membrane characteristics with regard to protein composition and functionality, contain numerous microvilli and are physically separated from the basolateral space by adherens and tight junctions (Figure 3). The multicellular apical lumens closely resemble the first clear indication of parenchymal organization in embryonic and regenerating liver (Ogawa et al., 1979; Stamatoglou et al., 1992). In vivo, such cellular rearrangement occurs transiently during development and regeneration (preceding plate formation) but appears to be the end stage of morphological differentiation during carcinogenesis (Stamatoglou et al., 1994). The formation of an elaborate, anastomosing canalicular network subsequent to the development of an acinar morphology was not apparent within the time span of our experiments, although some branching of the canalicular lumen was observed (Figure 8, B and C).

Because the transition from small apical lumens between two cells to larger multicellular (>5 cells) lumens occurs concomitantly with a decrease in the total number of apical lumens in the culture, and because serial confocal microscopy x-y sections reveal the clustering of apical lumens during this process, it is tempting to speculate that the large multicellular apical lumens may form, at least in part, via the merging of existing BC (Figures 4, F–N, and 5). This process has been named “cord hollowing,” where pockets of fluid-filled lumens occur at the lateral cell surfaces along the central axis of a multicellular cord, which then expand by virtue of ongoing delivery of apical vesicles, and, by an unknown mechanism, coalesce to form tubular structures (Lubarsky and Krasnov, 2003). The cord hollowing model is in agreement with the morphological stadia described in...
early studies on bile canalicular development in rat livers in vivo (De Wolf-Peeters et al., 1974; Luzzatto, 1981; Kanamura et al., 1990) and in primary cocultures with rat small hepatocytes and nonparenchymal cells (Sudo et al., 2004). Together, our data suggest that hepatocyte-derived ECM may contribute to multicellular patterning and bile canalicular surface remodeling and offer an experimentally controlled hepatic model system in which bile canalicular lumen morphogenesis can be induced and the underlying molecular mechanisms studied.

The remodeling of apical lumens seems to be regulated by distinct signaling pathways, including lipoxygenase-controlled ETA metabolism, the small GTPase Rho effector protein ROCK, its downstream target myosin-II ATPase, and p42/44 MAPK activity. Indeed, cell patterning, i.e., the formation of multilayering cell clusters, and apical lumen remodeling in HepG2 cultures grown on predeposited ECM is completely inhibited upon addition of ETA in an NDGA-sensitive manner (Figure 6). ETA stimulates signaling by ROCK, evidenced by increased phosphorylation of ML2C on serine-19 in a Y-27632-sensitive manner (Figure 7, A and B). Furthermore, the formation of multilayering cell clusters and remodeling of apical lumens can be induced in cell cultures grown on glass coverslips upon treatment of the cells with the specific ROCK inhibitor Y-27632 (Figure 4) or inhibitors of myosin-II heavy chain ATPase BDM and blebbistatin (Figure 7, F and G, and Supplemental Figure S3). Because the effects of myosin-II inhibition on cell reorganization and apical lumen remodeling could not be prevented with ETA, down-regulation of myosin-II ATPase activity likely occurs downstream of ROCK inhibition, consistent with ML2C being a target for ROCK. The remarkably similar effects on cell–cell organization and apical lumen dynamics after culture on predeposited ECM or inhibition of ROCK/myosin-II suggest that the ECM down-regulates ROCK, and, subsequently, myosin-II ATPase activity, to stimulate cell patterning and apical lumen remodeling. Presumably, the uncoated glass coverslip precludes cell clustering and apical lumen remodeling by promoting high ROCK and myosin-II activity (Wozniak et al., 2003; Olsen, 2004), which can be overcome by the deposition of sufficient amounts of ECM. Collagen-, laminin-, fibronectin-, or poly-L-lysine–coated coverslips failed to down-regulate ML2C phosphorylation and did not induce the morphogenic effects as observed on predeposited ECM (our unpublished data), underscoring the necessity of correct signaling cascades initiated by the extracellular environment. The composition of the HepG2-derived ECM and the responsible integrins that link the extracellular ECM to the intracellular ROCK signaling cascade remain to be investigated. Importantly, the inhibition or activation of ROCK and subsequent ML2C phosphorylation does not visibly affect the formation of apical lumens per se (i.e., those formed between 2 or 3 cells), indicating that apical lumen formation and subsequent apical lumen remodeling are controlled by distinct mechanisms.

Although the involvement of the ROCK–myosin-II signaling cascade in HepG2 cell organization and apical lumen remodeling seems evident, the mechanism by which myosin-II ATPase contributes to this process remains elusive. The formation of extended canalicular lumens most likely requires lateral surface dynamics and apical junction remodeling (Zegers et al., 2003; Paul and Beitel, 2005), and myosin-II may play a role in regulating the dynamics of adhesion receptors (Bertet et al., 2004; Delaunois-Dubrac et al., 2004; Shewan et al., 2005), consistent with its localization in HepG2 cells (Figure 7, C and D).

In addition to the involvement of ROCK and myosin-II, also p42/44 MAPK activity seems to be crucial for apical lumen remodeling, evidenced by the absence of canalicular lumen elongation in cell cultures grown on predeposited ECM in the presence of the specific MAPK signaling inhibitor PD98059 (Figure 8). Interestingly, in contrast to apical lumen remodeling, ECM-mediated cell multilayering was not affected by PD98059 treatment, suggesting that p42/44 MAPK signaling is involved in a step distal to that controlled by ROCK and myosin-II. We did not find evidence of ECM- or Y-27632–stimulated phosphorylation of p42/44 MAPK (Figure 8I), suggesting that basal MAPK activity is required.

Together, the data demonstrate that human HepG2 cells can be rapidly induced to adopt a organoid organization in an experimentally controlled manner and present lipoxygenase-controlled ETA metabolism, ROCK, myosin-II, and p42/44 MAPK as the first identified factors involved in hepatocyte-derived ECM-mediated multicellular patterning and bile canalicular lumen morphogenesis. Understanding the mechanisms that control this organoid organization and the consequent prospective to maintain liver-specific architecture and therewith correlated function of hepatocytes in culture is expected to advance the development of liver-related cell-based therapies, including the transplantation (genetically modified) liver cells, the development of liver tissue substitutes for implantation, and/or toxicological screening, and extracorporeal circuits consisting of live cells.

ACKNOWLEDGMENTS

We thank Erik de Vries for expert technical assistance, Karin Klappe for preparing the C6-NBD-SM, and Jetse Stokroos for scanning electron microscopy. S.v.I.J. is supported by the Royal Dutch Academy of Sciences (KNAW).

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