Regulatory Subunit I-controlled Protein Kinase A Activity Is Required for Apical Bile Canalicular Lumen Development in Hepatocytes

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Signaling via cAMP plays an important role in apical cell surface dynamics in epithelial cells. In hepatocytes, elevated levels of cAMP as well as extracellular oncostatin M stimulate apical lumen development in a manner that depends on protein kinase A (PKA) activity. However, neither the identity of PKA isoforms involved nor the mechanisms of the cross-talk between oncostatin M and cAMP/PKA signaling pathways have been elucidated. Here we demonstrate that oncostatin M and PKA signaling converge at the level of the PKA holoenzyme downstream of oncostatin M-stimulated MAPK activation. Experiments were performed with chemically modified cAMP analogues that preferentially target regulatory subunit (R) I or RII holoenzymes, respectively, in hepatocytes. The data suggest that the dissociation of RII- but not RII-containing holoenzymes, as well as catalytic activity of PKA, is required for apical lumen development in response to elevated levels of cAMP and oncostatin M. However, oncostatin M signaling does not stimulate PKA holoenzyme dissociation in living cells. Based on pharmacological and cell biological studies, it is concluded that RI-controlled PKA activity is essential for cAMP- and oncostatin M-stimulated development of apical bile canalicular lumens.

Hepatocytes perform most of the metabolic functions of the liver. For this, their asymmetric or polarized architecture is crucial. Thus, a sinusoidal or basolateral surface domain faces the space of Disse and allows the uptake/secretion of proteins and lipids from/into the blood, whereas a bile canalicular or apical surface domain faces the bile canaliculi and is equipped with proteins and lipids that facilitate bile secretion and protect the hepatocytes from the detergent-like action of biliary components. Loss of functional and/or structural hepatocyte polarity leads to hepatocyte dedifferentiation and liver disease. Understanding the cell biological and molecular principles that underlie hepatocyte polarity establishment and maintenance is important for the following reasons: (i) for developing therapeutic strategies to combat liver disease, and (ii) for the development of culture conditions that preserve hepatocyte differentiation in cell culture, e.g. for their application in bioartificial liver systems.

In vitro hepatocyte models have long suffered from limited or inefficient polarization. However, various cell models such as WIF-B (1), their canalicular network-forming derivatives (2), and improved culture conditions (see Refs. 3–8, for example) are currently available for the study of hepatocyte polarity in vitro (9). We and others have employed well differentiated human hepatoma HepG25 cells as a model system to study hepatocyte polarity. At the morphological level, HepG2 cells readily and reproducibly develop apical surface domains and bile canalicular lumens between neighboring cells as a function of time in culture (10–13). Moreover, following the deposition of extracellular matrix components, HepG2 cells spatially reorganize and display bile canalicular lumen morphogenesis that resembles the early organizational pattern during liver development (3). HepG2 cells therefore provide a suitable cell culture model for the study of hepatocyte polarity and bile canalicular lumen development (14).

The molecular mechanisms and signaling pathways that regulate hepatocyte morphogenesis and the formation of the apical surface and bile canalicular lumens are not well understood, but they include among others the interleukin 6 family cytokine oncostatin M (OSM) (15). OSM influences a variety of cellular events that contribute to hepatocyte differentiation during liver development, which include the transcription of HNF4α-regulated genes (16), metabolic functions (17), E-cadherin (18), and tight junction (19)-mediated cell-cell adhesion, and the development of apical-basal polarity (20–22). The transcriptional regulation of protein expression and the establishment of proper cell-cell adhesions allow bile canalicular lumen development.

The abbreviations used are: HepG2, human hepatoma cell line; 6-MBC-cAMP, 6′-mono-tert-butylcarbamoyladenosine-3′,5′-cyclic monophosphate; 8-Br-2′,3′-OMe-cAMP, 8-bromo-2′,3′-O-methyladenosine-3′,5′-cyclic monophosphate; 8-PIP-cAMP, 8-piperidinoadenosine-3′,5′-cyclic monophosphate; BC, bile canaliculus; BRET, bioluminescence resonance energy transfer; C, catalytic subunit; Ct,cAMP, dibutyryl cAMP; ERM, ezrin-radixin-moesin protein family; FSK, forskolin; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; p-8-Cl-cAMPS, 8-chloroadenosine-3′,5′-cyclic monophosphate; (p)-8-AHA-cAMP, 8-(6-aminohexylamino)adenosine-3′,5′-cyclic monophosphate; (p)-5,6-DCl-cBIMPS, 5,6-di-chloroimidazolodioxide riboside-3′,5′-cyclic monophosphorothioate; (p)-8-Cl-cAMP, 8-Chloroadenosine-3′,5′-cyclic monophosphorothioate; (p)-6-DCl-CBMP5, 5,6-di-chloroimidazolodioxide riboside-3′,5′-cyclic monophosphorothioate; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; h, human.

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5 The abbreviations used are: HepG2, human hepatoma cell line; 6-MBC-cAMP, 6′-mono-tert-butylcarbamoyladenosine-3′,5′-cyclic monophosphate; 8-Br-2′,3′-O-Me-cAMP, 8-bromo-2′,3′-O-methyladenosine-3′,5′-cyclic monophosphate; 8-PIP-cAMP, 8-piperidinoadenosine-3′,5′-cyclic monophosphate; 8-AHA-cAMP, 8-(6-aminohexylamino)adenosine-3′,5′-cyclic monophosphate; BC, bile canaliculus; BRET, bioluminescence resonance energy transfer; C, catalytic subunit; Ct,cAMP, dibutyryl cAMP; ERM, ezrin-radixin-moesin protein family; FSK, forskolin; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; p-8-Cl-cAMPS, 8-Chloroadenosine-3′,5′-cyclic monophosphorothioate; (p)-8-AHA-cAMP, 8-(6-aminohexylamino)adenosine-3′,5′-cyclic monophosphate; (p)-5,6-DCl-cBIMPS, 5,6-di-chloroimidazolodioxide riboside-3′,5′-cyclic monophosphorothioate; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; h, human.
PKA Isoforms in Cell Polarity

The first steps in the structural and functional development of apical bile canalicular plasma membrane domains and lumens are believed to involve the local remodeling of the lateral plasma membrane and cytoskeleton, the establishment of intercellular adhesion junctions, and the targeted supply of bulk membrane, fluid, and specific proteins and lipids that confer apical identity to the newly formed surface domain (reviewed in Ref. 23). Intracellular levels of cAMP play an important role in this process. Indeed, extracellular agents that stimulate cAMP production in the cells via adenyl cyclase promote membrane trafficking to the apical plasma membrane domain and apical surface development (23). cAMP/protein kinase A (PKA) stimulate several apical surface-directed trafficking pathways in hepatic cells, including those originating from the Golgi apparatus (24), from the basolateral surface (transcytosis) (24, 25), and from the subapical compartment/common endosome (13, 26). cAMP/PKA-stimulated trafficking from the subapical compartment/common endosome was demonstrated to be necessary for apical surface development (13, 26). Little is known about the downstream effectors of cAMP in the development of apical lumens. Although some effects of cAMP can be mediated by the Rap guanine nucleotide exchange factor Epac (27, 28), the primary intracellular target of cAMP is PKA. Following binding of cAMP to the regulatory (R) subunits of PKA holoenzymes, these dissociate from and allow the activation of the catalytic (C) subunits. Inhibition of PKA catalytic activity inhibits (cAMP-stimulated) apical membrane-directed trafficking and prevents apical lumen development (29). Interestingly, the process of PKA-controlled membrane trafficking and apical lumen development is activated by the extracellular cytokine OSM (20, 21). Thus, exposure of HepG2 cells to OSM activates PKA-dependent membrane trafficking to the apical plasma membrane domain and ensuing apical surface development, whereas the inhibition of apically directed trafficking by H89 prevents the stimulatory effect of OSM on cell polarity (20). However, how OSM and cAMP/PKA signaling pathways connect in the cell is not clear.

Cells express different PKA isoforms that are composed of distinct regulatory and catalytic subunits. It is not known whether cAMP and OSM require the enzymatic activity of the same PKA holoenzyme(s) to stimulate apical cell surface and lumen development. For instance, we have recently shown that OSM, but not cAMP analogues, stimulates the association of the PKA R subunit IIα with centrosomes (20, 22), suggesting that OSM and cAMP utilize PKA signaling differently. The purpose of this study was to identify the specific PKA holoenzyme(s) that mediates the cell polarity-stimulating effects of OSM and cAMP in HepG2 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human hepatoma HepG2 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 1-glutamine, 10% fetal calf serum, and penicillin/streptomycin, as described elsewhere (24). For immunofluorescence experiments, cells were plated onto ethanol-sterilized glass coverslips.

Determination of HepG2 Cell Polarity—The evaluation of polarity was performed as described elsewhere (12, 13). Briefly, HepG2 cells were plated onto coverslips for the indicated times and fixed with absolute ethanol (−20 °C) for 10 s, washed three times× with PBS, and subsequently incubated with TRITC-labeled phalloidin (100 ng/ml) and Hoechst 33528 (5 ng/ml) in PBS at room temperature for 30 min. The coverslips were then washed with PBS and mounted. The level of polarity was determined by counting the number of TRITC-phalloidin-positive bile canaliculi (BC) per 100 cells, and expressed as the ratio (BC/100 cells)×100%. At least 10 fields each containing >300 cells were counted using an epifluorescence microscope (Provis AX70, Olympus Corp. New Hyde Park, NY).

SDS-PAGE and Western Blotting—Cells were washed three times with ice-cold PBS and lysed in lysis buffer (PBS containing 1% Nonidet P-40 and protease inhibitors as follows: 1 μg/ml aprotinin, 100 μM benzamidine, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin A). After incubation on ice for 30 min, cells were homogenized in a syringe with a 27-gauge needle (20 strokes) and centrifuged for 10 min at 16,000 × g at 4 °C. Five microliters of the supernatant were processed for protein determination. Samples were then resuspended in sample buffer containing 2% SDS, 1% mercaptoethanol, 10% glycerol, 50 mM Tris, pH 6.8, 0.02% bromphenol blue, and boiled for 1 min. Equal amounts of proteins were separated on a 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked and incubated with mouse anti-phospho-p44/p42 MAPK and rabbit anti-MAPK antibodies (Cell Signaling Technology). Membranes were then washed three times for 5 min with PBS/Tween 20 (0.5%) and incubated with appropriate secondary antibodies (Westburg, Hillerod, Denmark) at room temperature for 45 min and processed for detection of immunoreactive signals using an Odyssey infrared imaging system (Westburg, Hillerod, Denmark), according to the manufacturer’s instruction.

Treatment of Cells with cAMP Analogues and Other Compounds—HepG2 cells were grown on coverslips and incubated with different PKA type I/II-specific activating/inhibiting cAMP analogues (Biolog, Bremen, Germany). For activation of PKA type I, the combination of 8-PIP-cAMP and 8-AHA-cAMP was used. For activation of PKA type II, the combination of 6-MBC-cAMP and (S)-5,6-DCI-cBIMPS was used (see “Results”). These activators, as well as dibutyl cAMP (Bt2cAMP, Calbiochem), were dissolved in Hanks’ balanced salt solution and diluted into culture medium to the final (combined) concentration of 1 mM. The PKA type I-specific inhibitory analogue (R)-8-Cl-cAMPS (see under “Results”) was dissolved in Hanks’ balanced salt solution and diluted in culture medium to the final concentration of 500 μM. Oncostatin M (Cell Concepts) was dissolved in serum-free medium and used at a final concentration of 10 ng/ml. KT5720 and H89 (Calbiochem) were dissolved in DMSO and added directly to the culture medium at a final concentration of 10 μM. Forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX), iso-proterenol, and PD98059 (Sigma) were used in combination at concentrations of 50 μM, 1 mM, 100 μM, and 50 μM, respectively. In some experiments cells were preincubated with PKA inhibitors KT5720 (10 μM) or H89 (10 μM) or the membrane-permeable adenyl cyclase inhibitor 2',3'-isopropylidene adenosine (2',3'-IA; 100
OSM Requires PKA Activity Downstream of p42/44 MAPK Phosphorylation in Order to Stimulate Cell Polarity Development—To visualize the stimulatory effect of 1) FSK/IBMX, 2) the stable cAMP analogue Bt$_2$cAMP, and 3) OSM on the development of apical lumens in HepG2 cells, cells were cultured for 3 days and incubated with either compound for 4 h. In addition, cells were treated with the cAMP analogue 8-Br-2'-O-Me-cAMP which, when used at appropriate concentrations, activates Epac but not PKA (32, 33). Two membrane-permeant ATP-binding site inhibitors, H89 and KT5720, were included in the incubations to determine PKA dependence. Following the incubation, cells were fixed and stained with TRITC-phalloidin (depicted in red) and Hoechst (depicted in blue) to label the subapical actin cytoskeleton and cell nuclei, respectively. The lack of a stimulatory effect of 8-Br-2'-O-Me-cAMP (Fig. 2A) supports that PKA mediates the effect of cAMP signaling on apical lumen development.

Whereas FSK/IBMX and Bt$_2$cAMP are well known to stimulate PKA activity in cells, the link between OSM and PKA signaling is not clear. Because the stimulatory effect of OSM on cell polarity development appears to be dependent on PKA activity (Fig. 2A) (cf. Ref. 20), we first examined whether OSM requires PKA activity to elicit a signaling response. In HepG2 cells, OSM binds to its receptor at the (basolateral) plasma membrane and stimulates the recruitment of the signal-transducing coreceptor gp130 into cholesterol-dependent membrane microdomains or rafts (20). A well characterized downstream target of gp130 is the Ras-Raf-MEK-p42/44 MAPK signaling pathway. Therefore, HepG2 cells were pretreated with PKA inhibitors and subsequently treated with OSM. Cells were then lysed, and proteins were subjected to gel electrophoresis and immunoblotting with antibodies raised against p42/44 MAPK and phospho-p42/44 MAPK. Fig. 2B shows that OSM phosphorylated p42/44 MAPK and that this was not inhibited by KT5720 or H89. Importantly, inhibition of p42/44 MAPK phosphorylation with the MEK inhibitor PD98059 effectively abolished the stimulatory effect of OSM, but not Bt$_2$cAMP, on cell polarity development (Fig. 2C). Treatment of the cells with PD98059 alone for the duration of the experiments did not affect the number of apical lumens (Fig. 2E). These data show that OSM/MEK-p42/44 MAPK signaling requires PKA activity downstream of MEK-p42/44 MAPK to stimulate cell polarity development, whereas cAMP/PKA does not require OSM/MEK/MAPK signaling to stimulate cell polarity development.

Inhibition of Adenylyl Cyclase Does Not Prevent OSM-stimulated Cell Polarity Development—To examine which elements of the cAMP/PKA signaling pathway are required for the stimulatory effect of OSM on apical lumen development, cells were preincubated with the membrane-permeable adenylyl cyclase inhibitor 2',3'-IA (34, 35) or buffer (control). Subsequently, cells were treated with OSM or FSK for 4 h. The adenylyl cyclase inhibitor was kept present during all incubations where appropriate. Cells were then fixed and fluorescently labeled to determine the ratio of BC/100 cells as a quantitative measure for cell polarity (see “Experimental Procedures”). As shown in Fig. 2D, 2',3'-IA prevented the stimulatory effect of FSK on apical lumen development, suggesting that 2',3'-IA indeed inhibits adenylyl cyclase activity.
inhibited cAMP production (34, 35). In contrast, 2',3'-IA did not prevent OSM from stimulating apical lumen development. Treatment of the cells with 2',3'-IA alone for the duration of the experiment did not affect the number of apical lumens (Fig. 2E). Given that OSM-stimulated apical lumen development requires PKA activity, and in agreement with the observation that OSM does not increase cellular cAMP levels in HepG2 cells (20), these data suggest that OSM/MAPK and cAMP/PKA signaling pathways connect independent of de novo cAMP production. Because catalytic activity of PKA is evidently required for OSM to stimulate cell polarity development (cf. Fig. 2A) (20), it is suggested that the OSM/MAPK connects with PKA signaling at the level of the holoenzyme.

**Modified cAMP Analogues and BRET Are Suitable Tools for Monitoring the Activation of Distinct PKA Holoenzymes in Living Cells**—Distinct PKA holoenzymes can be distinguished by their tetrameric composition of regulatory (RI or RII) and catalytic subunits. To determine whether type I or type II PKA mediates cell polarity development in response to either cAMP analogues or OSM, we employed chemically modified and membrane-permeable analogues of cAMP that bind with different affinity to the A and B sites of the regulatory subunits (site selectivity). In vitro assays, the combination of appropriate couples of analogues, i.e. 8-PIP-cAMP/8-AHA-cAMP or (S)-5,6-DCI-cBIMPS/6-MBC-cAMP, cause the selective dissociation of PKA type I (RI/C) or II (RII/C), respectively (36, 37), which can be taken as a measure for specific PKA holoenzyme activity. We first verified that these analogue combinations also cause the dissociation of specific holoenzymes in living HepG2 cells. For this we used a BRET

![Figure 2. Involvement of PKA in apical lumen development in HepG2 cells.](image-url)
PKA Isoforms in Cell Polarity

We then investigated the effects of these analogue combinations on apical surface and lumen development. For this, HepG2 cells were cultured for 3 days and subsequently incubated for 1 h in medium supplemented with 8-PIP-cAMP/8-AHA-cAMP, (S)-5,6-DCI-cBIMPS/6-MBC-cAMP, or vehicle. Cells were then fixed and fluorescently labeled to determine the ratio BC/100 cells as a measure for cell polarity (see “Experimental Procedures”). Fig. 5A shows that treatment of the cells with the RI-prefering cAMP analogues stimulated cell polarity development similar to that observed with Bt2cAMP or FSK/IBMX, and this was inhibited when cells were pretreated with H89. In contrast, the RII-selective cAMP analogues did not stimulate polarity development (Fig. 5A). These data indicate that the dissociation and resultant enzymatic activity of type I PKA, rather than that of type II PKA, are involved in apical lumen development in HepG2 cells.
whereas it does not prevent FSK/IBMX- or Bt2cAMP-stimulated CAMPS for 1 h. Where indicated, cells were pretreated with either H89 or a stable inhibitor of PKA, (p)-8-Cl-cAMPS, occupying cAMP-binding sites at the regulatory subunit of PKA thus preventing the holoenzyme from dissociation and, consequently, kinase activation. Although (p)-8-Cl-cAMPS has been reported to function as a site-selective competitive inhibitor of PKA with preference for type I PKA (38), we first verified this preference in living HepG2 cells using the BRET assay as described above. As shown in Fig. 4C, (p)-8-Cl-cAMPS prevents dissociation of the RI/C holoenzyme induced by FSK/IBMX and Bt2cAMP, whereas it does not prevent FSK/IBMX- or Bt2cAMP-stimulated dissociation of RII/C holoenzymes (Fig. 4D). We then pretreated HepG2 cells with (p)-8-Cl-cAMPS or buffer, and we subsequently exposed the cells to Bt2cAMP or OSM. When cells were treated with the inhibitory analogue, it was kept present during the subsequent incubations. Cells were fixed and fluorescently labeled with TRITC-phalloidin and Hoechst, and the ratio of BC/100 cells was determined as an accurate measure of cell polarity (see “Experimental Procedures”).

To obtain further evidence for the involvement of type I PKA, we employed a potent membrane-permeable and metabolically stable inhibitor of PKA, (p)-8-Cl-cAMPS, occupying cAMP-binding sites at the regulatory subunit of PKA thus preventing the holoenzyme from dissociation and, consequently, kinase activation. Although (p)-8-Cl-cAMPS has been reported to function as a site-selective competitive inhibitor of PKA with preference for type I PKA (38), we first verified this preference in living HepG2 cells using the BRET assay as described above. As shown in Fig. 4C, (p)-8-Cl-cAMPS prevents dissociation of the RI/C holoenzyme induced by FSK/IBMX and Bt2cAMP, whereas it does not prevent FSK/IBMX- or Bt2cAMP-stimulated dissociation of RII/C holoenzymes (Fig. 4D). We then pretreated HepG2 cells with (p)-8-Cl-cAMPS or buffer, and we subsequently exposed the cells to Bt2cAMP or OSM. When cells were treated with the inhibitory analogue, it was kept present during all subsequent incubations. Fig. 5B shows that pretreatment of the cells with (p)-8-Cl-cAMPS effectively prevented the stimulating effect of Bt2cAMP as well as that of OSM. Treatment of the cells with (p)-8-Cl-cAMPS alone for the duration of the experiment did not affect the number of apical lumens (Fig. 5B). These data support that the enzymatic activity of type I PKA is involved in OSM- and cAMP-mediated polarity development of hepatoma HepG2 cells.

OSM Does Not Cause PKA Holoenzyme Dissociation in Living HepG2 Cells—Using fluorescence-based PKA activity assay in cell lysates, we have previously shown that OSM, in contrast to Bt2cAMP, does not result in overall PKA activation (20). However, it was demonstrated that OSM, but not Bt2cAMP, stimulates the association of PKA RIIα subunit with sub-apically positioned centrosomes. This requires MEK/MAPK activity but does not depend on catalytic PKA activity, and the OSM-stimulated association of RIIα with centrosomes is required for OSM to enhance apical lumen development (20, 22). These data thus suggested that the OSM effect mediated via type I PKA is more likely to be related to a mobilization of RIIα to its target site, thus indirectly affecting the efficiency of local PKA activity, than to an overall stimulation of its activity. To examine whether we could detect a stimulation of type II or type I PKA activity in response to OSM in live cells, thus preserving the subcellular distribution of PKA holoenzymes, we used the BRET assay as described above. HepG2 cells expressing RIIα/C or RIIα/C sensors were treated with either OSM, the adenylyl cyclase-activating compound isoproterenol, Bt2cAMP, or buffer at 37 °C, and the BRET signal was measured. As shown in Fig. 5, C and D, where Bt2cAMP caused the dissociation of the RIIα/C and RIIα/C holoenzymes, isoproterenol only affected the dissociation of RII/C holoenzymes, whereas OSM was without significant effect. Together, these data indicate that, in contrast to elevated [cAMP]p, OSM does not stimulate PKA holoenzyme dissociation and activation in living cells.

DISCUSSION
cAMP and PKA have been implicated in the dynamics of functional apical cell surface domains and apical lumens in various epithelial cell types, including hepatocytes (reviewed in Ref. 23). PKA is a holoenzyme complex composed of regulatory and catalytic subunits, distinguishing type I (RI/C) and type II (RII/C) isoforms. The purpose of this study was to investigate whether specific PKA holoenzymes are involved in the development of the apical bile canalicular surface and lumen in...
human HepG2 cells. For this, we employed chemically modified pairs of cAMP analogues that have been reported to preferentially bind to RI or RII subunits in in vitro assays with purified PKA subunits. Using the recently developed BRET-based biosensor (30), we demonstrate that specific combinations of modified cAMP pairs can also be used in living cells to selectively dissociate either RI or RII holoenzymes. Furthermore, our assay showed that FSK/IBMX and Bt2cAMP cause the dissociation of both RI and RII holoenzymes, whereas isoproterenol causes the selective dissociation of RII holoenzymes in HepG2 cells. Finally, our assay demonstrated that the cAMP antagonist (R<sub>i</sub>)-8-Cl-cAMPS preferentially prevented the dissociation of RI/C holoenzymes following treatment of the cells with Bt2cAMP. Chemically modified cAMP analogues are therefore a useful tool to determine the selective involvement of PKA holoenzymes in processes occurring in living cell systems.

Our combined data with the site-selective cAMP analogues (PKA agonists and antagonists) and inhibitors of the ATP site of the catalytic subunits indicate that [cAMP], stimulates cell polarity development in a manner that requires the dissociation of type I PKA holoenzymes and subsequent catalytic activity.

We have recently demonstrated that OSM signaling stimulates apical lumen development in a p42/44 MAPK- and also PKA-dependent manner (20, 22). Here we demonstrate that the stimulatory effect of OSM on apical lumen development requires the enzymatic activity of PKA downstream of MAPK phosphorylation and does not require de novo MAPK production, suggesting that OSM and PKA signaling join at the level of the PKA holoenzyme. Experiments with (R<sub>i</sub>)-8-Cl-cAMPS, a cAMP antagonist that preferentially binds to RI subunits but without causing holoenzyme dissociation, suggest that, similar to cAMP, the stimulatory effect of OSM on apical lumen development requires type I PKA holoenzymes. However, in contrast to cAMP, OSM signaling does not stimulate the dissociation of type I (or type II) PKA holoenzymes. It thus appears that OSM stimulates apical lumen development through MEK/MAPK and requires a base level of PKA activity that is under control of RI regulatory subunits. Taken together, we conclude that RI-controlled PKA activity is essential for cAMP- and OSM-mediated apical lumen development in HepG2 cells.

In a previous study, we showed that treatment of HepG2 cells with OSM, but not Bt2cAMP, stimulated the association of RIIα with centrosomes (20–22). This required MAPK phosphorylation but not the enzymatic activity of PKA. The recruitment of RIIα at centrosomes, but not the enzymatic activity of PKA, was shown to be required for the polarized subapical positioning of the centrosome (22). Importantly, displacing RIIα from centrosomes effectively prevented the stimulatory effect of OSM, but not that of cAMP, on apical lumen development (21, 22). By combining these data with the data presented in this study, it is suggested that the OSM effect on apical lumen development, mediated via PKA, includes a mobilization of RIIα to a target site in the subapical area (20, 22) and, in addition, includes a requirement for the base level of PKA type I activity (this study) downstream of or parallel to OSM-stimulated RIIα association with centrosomes. Our current working model is depicted in Fig. 6.

Interestingly, the ERM proteins radixin and ezrin predominantly associate with the bile canicular surface (39), where they act as scaffolding proteins and control apical cytoskeleton dynamics and apical membrane structure. Radixin and ezrin function as protein kinase A-anchoring proteins (AKAPs) and recruit both PKA RI and RII subunits (40). Loss of radixin, the predominant ERM protein in hepatocytes, inhibits bile canicular membrane structure and function (41) and causes hyperbilirubinemia (42). It will be of interest to investigate in future studies the involvement of ERM proteins in PKA signaling-dependent apical bile canicular surface and lumen development, and to unravel the specific contributions of type I and type II PKA in this process.

The study of the PKA RI subunit and its involvement in liver development in model organisms is difficult. The targeted disruption of RIIα and subsequent unregulated PKA activity in mice leads to failure of embryos at the earliest stages of development. Interestingly, one of the most prominent features of these mice is the absence of a definitive heart tube (43, 44). Heart tube development involves the establishment of cell polarity and the formation of an apical lumen (45–49). It will be of interest to investigate whether RI-controlled PKA regulates common cellular processes in different organs such as the heart and liver that are important for the development of apical lumens in general.

REFERENCES

1. Ihrke, G., Neufeld, E. B., Meads, T., Shanks, M. R., Cassio, D., Laurent, M., Schroer, T. A., Pagano, R. E., and Hubbard, A. L. (1993) J. Cell Biol. 123,
