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AKAP350 is involved in the development of apical “canalicular” structures in hepatic cells HepG2

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Abstract

Hepatocytes are epithelial cells whose apical poles constitute the bile canaliculi. The establishment and maintenance of canalicular poles is a finely regulated process that dictates the efficiency of primary bile secretion. Protein kinase A (PKA) modulates this process at different levels. AKAP350 is an A-kinase anchoring protein that scaffolds protein complexes involved in modulating the dynamic structures of the Golgi apparatus and microtubule cytoskeleton, facilitating microtubule nucleation at this organelle. In this study, we evaluated whether AKAP350 is involved in the development of bile canaliculi-like structures in hepatocyte derived HepG2 cells. We found that AKAP350 recruits PKA to the centrosomes and Golgi apparatus in HepG2 cells. De-localization of AKAP350 from these organelles led to reduced apical cell polarization. A decrease in AKAP350 expression inhibited the formation of canalicular structures and impaired F-actin organization at canalicular poles. Furthermore, loss of AKAP350 expression led to diminished polarized expression of the p-glycoprotein (MDR1/ABCB1) at the apical “canalicular” membrane. AKAP350 knock down effects on canalicular structures formation and actin organization could be mimicked by inhibition of Golgi microtubule nucleation by depletion of CLIP associated proteins (CLASPs). Our data reveal that AKAP350 participates in mechanisms which determine the development of canalicular structures as well as accurate canalicular expression of distinct proteins and actin organization, and provide evidence on the involvement of Golgi microtubule nucleation in hepatocyte apical polarization.

Keywords

AKAP350; PKA; bile canaliculus; actin cytoskeleton; CLASP

INTRODUCTION

A-kinase-anchoring proteins (AKAPs) are a family of functionally heterogeneous proteins, which interact with PKA regulatory subunits and assemble multiprotein signal transduction complexes. Besides containing distinct protein interacting domains, AKAPs contain subcellular targeting domains, which position these complexes at specific sites in the cell

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(Wong and Scott, 2004). AKAP350 belongs to a group of AKAPs derived from a multiple spliced gene in chromosome 7 (AKAP9), which includes AKAP450/CG-NAP and yotiao (Schmidt et al., 1999; Takahashi et al., 1999; Witczak et al., 1999). AKAP350 localizes to the centrosomes and the Golgi apparatus, thus providing a scaffold for the targeted localization of its interacting proteins.

AKAP350 contains two PKA-R_{II} regulatory subunit interacting motifs (Schmidt et al., 1999), and binding domains for multiple signalling and cytoskeleton-associated proteins, including the nerve growth factor activated protein kinase PKN, protein phosphatases PP1 and PP2A (Takahashi et al., 1999), phosphodiesterase 4D (Tasken et al., 2001), calmodulin (Gillingham and Munro, 2000), the components of the γ -tubulin ring complex GCP2 and GCP3 (Takahashi et al., 2002), the Ran GTPase (Keryer et al., 2003), the cyclin dependent kinase 2 (Nishimura et al., 2005), different members of the chloride intracellular channels (Shanks et al., 2002b; Berryman and Goldenring, 2003), the transforming acidic coiled-coil protein TACC3 (Steadman et al., 2002), the cdc42 interacting protein 4 (CIP4) (Larocca et al., 2004), and the RNA stress granules-associated proteins caprin-1 and CCAR1 (Kolobova et al., 2009). By assembling some of these proteins at the centrosomes, AKAP350 is involved in the nucleation of centrosomal microtubules and the modulation of centrosome duplication during cell cycle progression (Takahashi et al., 2002; Keryer et al., 2003b; Nishimura et al., 2005). Non-centrosomal AKAP350 has an important role in interphase cells, as well. AKAP350 participates in microtubule nucleation at the Golgi apparatus (Rivero et al., 2009), microtubule growth (Larocca et al., 2006) and is involved in integrin-induced T cell migration (El Homasany et al., 2005), stress granules formation (Kolobova et al., 2009) and maintenance of Golgi apparatus structure (Larocca et al., 2004).

Hepatocytes are non-columnar epithelial cells which establish their apical poles at the sites of intercellular contact. The apical poles in hepatocytes constitute the bile canaliculi, membrane structures responsible for primary bile secretion. Proper bile secretion depends on the differential distribution of lipids and proteins in the basolateral (sinusoidal) and apical (canalicular) membrane. The organization of distinct membrane domains is achieved by selective mechanisms of sorting, trafficking, insertion, retention, and recycling of the hepatocyte membranes components. The precision of these mechanisms depends on the integrated function of the Golgi apparatus, the apical and basal recycling endosomes and the cytoskeleton and its associated proteins (Decaens et al., 2008; Weisz and Rodriguez-Boulant, 2009). Among the multiple proteins that coordinate the different determinants of hepatocyte polarity, PKA regulates the formation and composition of the bile canaliculi by stimulating the polarized trafficking of the canalicular membrane components (Zegers and Hoekstra, 1997; van IJendoorn and Hoekstra, 2000; Kipp and Arias, 2000; Gradilone et al., 2003). HepG2 cells develop apical structures which resemble bile canaliculi, i.e., they are formed by the lateral membrane between two cells and sealed by tight junctions, they express the canalicular ATP binding cassette (ABC) transporters, and they are delimited by a dense ring of F-actin (Decaens et al., 2008). Previous studies in HepG2 cells indicate that interaction of PKA-R_{II} with its anchoring proteins is necessary for the efficient sorting of distinct canalicular proteins and lipids (Wojtal et al., 2006) and for oncostatin M-stimulated canalicular development (Wojtal et al., 2007). Nevertheless, the identities of the AKAP(s) and protein complexes implicated have not been unravelled so far. In the studies reported here, we have evaluated the role of AKAP350 in hepatocyte apical polarization, by examining the effects of loss of AKAP350 function on the development of canalicular structures in HepG2 cells. Our results indicate that AKAP350 expression is necessary for normal development of canalicular structures and apical actin organization and for proper canalicular localization of distinct proteins, thus positioning AKAP350 as an excellent candidate for facilitating PKA function in these processes. Furthermore, our studies suggest

that the mechanism mediating AKAP350 dependent apical polarization involves microtubule nucleation at the Golgi apparatus.

MATERIALS AND METHODS

Cells

HepG2 cells (obtained from ATCC) were grown on plastic dishes in DMEM with 4.5 g of glucose per liter, supplemented with 10% fetal calf serum and antibiotics. Media was changed every other day. Cells were trypsinized after reaching 80% confluence. For immunofluorescence microscopy analysis, the cells were grown on glass coverslips and, at the end of the experiments, washed with PBS, and fixed in 4% paraformaldehyde in most experiments, or in 100 % methanol for assessment of centrosomal localization. For protein expression analysis by immunoblotting, the cells were washed twice with cold PBS, scraped and pelleted at $200 \times g$, for 3 minutes at 4°C.

Reduction of AKAP350 and CLIP associated proteins (CLASPs) expression by siRNA

The 21 nucleotides RNA duplexes (siRNA) used for decreasing AKAP350 expression were designed and synthesized following the guidelines described by Elbashir et al. (2001), as we previously described (Larocca et al., 2004). siRNA1 and siRNA2 specifically target human AKAP350 nucleotides 10917 to 10937 (AAATCCCTTGCCAGCACATGA) and 9397 to 9417 (AAGCAAGAACTAGAACGAGAA), respectively. The control siRNA (siRNA SC) was designed scrambling the nucleotides of the siRNA1 target sequence (AACTATGCCACCGTGACCTAA). In order to decrease CLASP1 and CLASP2 (CLASP1/2) expression, we used a mix of siRNAs designed by Mimori-Kiyosue et al. (2005). The siRNAs were synthesized using the Ambion's silencer™ siRNA Kit.

HepG2 cells were grown to 70% confluence and trypsinized. 2.5×10^5 cells were seeded in 35-mm plastic Petri dishes. Lipofectamine siRNAmix (Invitrogen) preincubated with the siRNA1 or the siRNA SC was added to the cells, following the manufacturer instructions. All the experiments were performed 48 hours after transfection, and the specific decrease in AKAP350 or CLASP1/2 expression was verified by immunoblotting in every case.

AKAP350 CTD-GFP expression

The AKAP350(3258–3524) domain was cloned into pEGFP-C2 (Clontech) using *EcoRI* and *SaII* sites, generating a construct coding for GFP fused to the carboxi-terminal domain of AKAP350 (AKAP350 3258–3524) at its amino-terminus (AKAP350 CTD-GFP). HepG2 cells were transfected by electroporation, as previously described (Kumar et al., 1994). Briefly, a suspension of 2.5×10^6 cells containing 30 µg of the pEGFP-AKAP350 CTD, or the pEGFP with no insert, were subjected to an electric pulse of 400 V/250 µF. 5×10^5 cells were seeded in 35 mm plastic Petri dishes. The cells were fixed 16 or 72 hours after transfection, and analyzed by immunofluorescence confocal microscopy.

Cytochalasin D recovery experiments

Cells were incubated with 2 µM cytochalasin D for 60 minutes, which was first established as the lowest dose that completely disrupts the pericanalicular F-actin cytoskeleton in HepG2 cells. After the incubation, the cells were washed three times with PBS, and re-incubated with cytochalasin-free media (recovery). Cells were fixed before and immediately after cytochalasin D treatment (Basal and Cytochalasin groups, respectively), and after different periods of recovery. We found that the maximal recovery of canalicular F-actin in the control cells was at 60 minutes, so we chose this period of incubation to analyze the effects of AKAP350 or CLASP1/2 depletion on canalicular structures composition.

Immunoblotting

Pelleted HepG2 cells were resuspended in 1% Triton X100/PBS pH 7.4 with protease inhibitors and subjected to two freeze(−80 °C)-thaw cycles. Lysates were then centrifuged at $1000 \times g$ for 5 minutes, and the clear supernatants were used. Total protein concentration in the solubilised membranes was measured according to Lowry et al. (1951). Solubilized membranes were heated 10 minutes at 70 °C in sample buffer (20 mM Tris, pH 8.5, 1% SDS, 400 μ M DTT, 10 % glycerol), and samples containing equal amounts of proteins were subjected to 4%/10% discontinuous or 10% SDS-polyacrylamide gel electrophoresis, for analysis of AKAP350/ABCB1/ABCC2/actin or CLASP expression, respectively. The proteins in the 4 % gel were transferred to nitrocellulose membranes (Amersham), while the 10% gel proteins were transferred to polyvinyl difluoride membranes (Perkin Elmer Life Sciences). Blots were blocked with 5% nonfat dry milk in PBS, 0.3% Tween-20 (PBS-Tween). Nitrocellulose blots were then probed with the primary antibody mouse anti-AKAP350 (1:500), and polyvinyl difluoride membranes were probed with rabbit anti-CLASP1 (1:1000) or rabbit anti-CLASP2 (1:500) (Efimov et al., 2007), mouse anti- β -actin (1:2000, Sigma), mouse anti-multidrug resistance protein 2 (MRP2/ABCC2) (1:1000, Zymed), rabbit anti-p-glycoprotein (MDR1/ABCB1) (1:1000, Santa Cruz) or mouse anti- α -tubulin (1:5000, Sigma). Polyclonal anti-CLASP1 antibodies were provided by Dr. F. Severin (Dresden) and anti-CLASP2 antibodies, by Dr. I. Kaverina (Vanderbilt University, Nashville TN). The blots were then washed and incubated with the horseradish peroxidase-conjugated corresponding secondary antibodies, and bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Autoradiographs were obtained by exposing the blots to Kodak XAR film. The bands were quantitated by densitometry using the NIH Image J program. In preparing the figures, adjustments of brightness and contrast were applied to the entire images for the sake of clarity.

Immunofluorescence and Confocal Microscopy

Fixed cells were permeabilized and blocked with 0.3% Triton X-100/1% Bovine Serum Albumin/PBS pH 7.4 for 10 minutes, and then incubated simultaneously with rabbit anti-AKAP350A (1:250), rabbit anti-ABCB1 (1:100), rabbit anti-R Π α (Santa Cruz) (1:100), rabbit anti-GOLPH4 and mouse anti-AKAP350 (1:80), mouse anti-ABCC2 (1:200), mouse anti-GM130 (1:100), mouse anti- γ -tubulin (1:500) for 2 hours at room temperature. After washing, coverslips were incubated for 1 hour with the corresponding Alexa 488, Alexa 555 and Alexa 633-conjugated secondary antibodies and/or Alexa 555 or Alexa 633 conjugated phalloidin (Molecular Probes) diluted in the blocking buffer. Cover slips were washed, incubated with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) (50 μ M) for 10 minutes, and mounted with ProLong (Molecular Probes). Fluorescence localization was detected by laser confocal microscopy (Nikon C1SiR with inverted microscope Nikon TE200). For quantitative analysis, serial optical sections of the staining in the Z axis were collected by laser confocal microscopy in 0.3 μ m steps. The Z-stacks for each field were built, and the projections of the stacks obtained using the corresponding Image J tools. For figures presentation, contrast and brightness adjustments were equally applied to the entire images using Adobe Photoshop software for the sake of clarity.

Analysis of Golgi and centrosomal localization of proteins

Golgi PKA and AKAP350—The staining of the cis-Golgi proteins GM130 or GOLIM4 (GOLPH4) were used to identify Golgi structures. Localization of AKAP350 and PKA at the Golgi was estimated by measuring Pearson's colocalization (Rr) and Mander's (M) coefficients between AKAP350 or PKA and GOLPH4 or GM130 channels, using Image J colocalization plug-in. In order to estimate the fraction of PKA or AKAP350 which localizes at the Golgi apparatus, " M_{PKA} " or " $M_{AKAP350}$ " were calculated using GOLPH4 or

GM130 staining at the Golgi apparatus to set the threshold. Thus, M measures the fraction of PKA or AKAP350 fluorescence which colocalizes with Golgi markers. AKAP350 displacement from the Golgi apparatus by AKAP350 CTD expression was verified by comparing R_r (AKAP350:GOLPH4) and $M_{AKAP350}$ between cells which express (AKAP350 CTD) or do not express (control) AKAP350 CTD-GFP. The effect of the displacement of AKAP350 from the Golgi apparatus on PKA localization at this organelle was analyzed by comparing R_r (PKA:GM130) and M_{PKA} between AKAP350 CTD-GFP and control cells. For the sake of clarity, results were also presented as percentage of AKAP350 or PKA present at the Golgi.

Centrosomal PKA—Similarly to Golgi PKA localization, “ R_r ” and “ M ” coefficients were measured to estimate centrosomal PKA localization. Additionally, centrosomes defined by γ -tubulin staining and the boundary of the cells assessed by differential interference contrast (DIC) were outlined for each analyzed cell by using the drawing tools in the Image J program. The total fluorescence intensity in centrosomes and total cell defined areas were determined in the channels corresponding to AKAP350 or PKA staining. With these measurements, we calculated the percentage of PKA associated fluorescence present at the centrosomes for every cell. Due to centrosomal morphology, we found that this morphometric analysis was more accurate than M calculation for determining the fraction of PKA fluorescence associated with this organelle. The effect of the displacement of AKAP350 from the centrosomes on PKA localization was analyzed by comparing the different parameters in control and AKAP350 CTD cells.

Assessment of Apical Accumulation of Glutathion-Methylfluorescein

Functional experiments were performed as previously described (Roma *et al.*, 2000). Control and AKAP350 KD live cells were loaded with 5-Chloromethylfluorescein diacetate (CMFDA) (2.5 μ mol/L) for 10 min at 37 °C. CMFDA is taken up by hepatocytes by passive diffusion across the basolateral membrane, and metabolized to glutathion-methylfluorescein (GS-MF) by hepatocyte esterases and glutathione S-transferases. GS-MF is actively secreted by the organic anion transporter ABCC2 to the canaliculus, where it concentrates. To quantify the presence of functional canaliculi we counted the number of fluorescent vesicle-like structures located in between two cells, and related it to the total number of cells.

Evaluation of HepG2 cell polarity

Determination of the degree of HepG2 polarity was performed as previously described (Zegers and Hoekstra, 1997). Cells were fixed and actin microfilaments, ABCC2, ABCB1 and nuclei were stained as described above. Canalicular poles were identified by the presence of structures located in between two or more cells with dense staining for F-actin, ABCC2 or ABCB1. The identification of canalicular structures using each marker was confirmed by the presence of a second marker in the identified structures. The cells were counted by determining the number of nuclei. The degree of polarity was determined and expressed as the ratio [number of canalicular structures/100 cells], or as the number of cells containing canalicular structures (polarized cells), when cells were transfected with pEGFP constructs. At least 500 cells distributed in 5–7 fields containing 50 to 100 cells each were counted per experiment.

Morphometric analysis of canalicular structures

Canalicular actin—Canalicular actin cytoskeleton was identified as the dense F-actin staining located in between two or more HepG2 cells. The boundary of the canalicular actin cytoskeleton and that of the cell itself was outlined based on the phalloidin staining using a freehand selection option in ImageJ software. The canalicular and total fluorescence

intensity were measured using the same program, and the percentage fluorescence present at the canalicular poles was calculated using these measurements. This parameter estimates the fraction of cell F-actin localized at the canalicular structures.

Canalicular protein localization—Cells were fixed and dual stained for actin and either ABCC2 or ABCB1. Colocalization studies were performed by calculating the Pearson's coefficient (R_r) between the ABCC2 or ABCB1 and the actin channels Z-stacks, using the Image J colocalization plug-in. For these studies, canalicular actin staining was used to set the threshold in the actin channel. Thus, we specifically assessed the colocalization of ABCC2 or ABCB1 with canalicular actin. Canalicular ABCC2 or ABCB1 localization in polarized cells was estimated by relating the total canalicular intensity of fluorescence to the total intensity of fluorescence in the polarized cells, as described above for F-actin.

Statistical Analysis—Data are expressed as mean $\pm$ s.e.m. Significance was determined using a paired Student's t-test for comparisons between experiments, and nonparametric Mann-Whitney rank sum test for comparisons inside each experiment; $p < 0.05$ was considered statistically significant.

RESULTS

AKAP350 is expressed at the Golgi apparatus and subapical centrosomes in HepG2 polarized cells, where it recruits $R_{II\alpha}$ -PKA

Previous studies demonstrated AKAP350 mRNA expression in human liver tissue (Schmidt et al., 1999), and we have confirmed its expression at the protein level in rabbit and human liver (data not shown). Wotjal *et al.* reported that AKAP350 is expressed in HepG2 cells (Wotjal et al., 2006). Thus, we first investigated AKAP350 localization in HepG2 cells by immunofluorescence, using a polyclonal antibody, which specifically recognizes AKAP450 and AKAP350A (Shanks et al., 2002b) (Fig. 1). Antibodies against the *cis*-Golgi protein GM130 and γ -tubulin were employed to stain the Golgi apparatus and the centrosomes, respectively. Confirming previous results in other epithelial cell lines, AKAP350 localized at the Golgi apparatus (Fig. 1A) and centrosomes (Fig. 1B, arrows) in HepG2 cells. $R_{II\alpha}$ is the most ubiquitous R_{II} subunit of PKA. Considering that the subcellular organization of $R_{II\alpha}$ -PKA protein complexes is controlled by its interaction with AKAPs (Wong and Scott, 2004), we analyzed $R_{II\alpha}$ -PKA colocalization with AKAP350 in HepG2 cells. Phalloidin staining was used to identify canalicular structures (Fig. 1C, chevrons). Our results showed that $R_{II\alpha}$ -PKA and AKAP350A colocalized at the Golgi apparatus (Fig. 1C, arrowheads) and at sub-apical centrosomes (Fig. 1C, arrows) in polarized cells.

AKAP350 domains targeting the scaffold to the centrosomes and to the Golgi apparatus are located in the carboxyl-terminal domain of the protein (Gillingham and Munro, 2000; Shanks et al., 2002a). To analyze the effect of displacing AKAP350 from these organelles on PKA localization, we prepared a construct for expression of the Golgi and centrosomal targeting domains of AKAP350 (AKAP350(3258–3524)) as a GFP chimeric protein (EGFP-AKAP350 CTD). GM130 or GOLPH4 and γ -tubulin were used as Golgi and centrosomal markers, respectively. Previous studies demonstrated that high levels of expression of AKAP350 centrosomal targeting domain leads to AKAP350 release from the centrosomes (Gillingham and Munro, 2000; Keryer et al., 2003b; Larocca et al., 2006). In agreement with these results, cells expressing high levels of AKAP350 CTD-GFP, where the GFP labelling was not only restricted to centrosomes, showed a significant loss of AKAP350 centrosomal staining (Fig. 2A–B arrows). Similar effects were observed with AKAP350 staining at the Golgi apparatus (Fig. 2B, arrowheads). Quantitative analysis of the images confirmed that cells expressing high levels of AKAP350 CTD-GFP have a 55 % decrease in the percentage

levels of Golgi associated AKAP350 fluorescence (control, 45 ± 4 ; AKAP350 CTD, 18 ± 3 , $p < 0.01$). AKAP350 delocalization was accompanied with alteration of the Golgi apparatus morphology (Fig. 2B and 2D). These results are consistent with our previous findings, which showed that reduction of AKAP350 levels leads to loss of Golgi stacking and dispersal of Golgi vesicles (Larocca et al., 2004). We next analyzed if the displacement of AKAP350 from the centrosomes and the Golgi apparatus interfered with $R_{II\alpha}$ -PKA localization at these organelles (Fig. 2C–D). Analysis of Pearson's correlation (R_r) coefficient in AKAP350 CTD and control cells showed that delocalization of AKAP350 led to a significant decrease in $R_{II\alpha}$ -PKA colocalization with α -tubulin (control, 0.242 ± 0.012 ; AKAP350 CTD, 0.040 ± 0.001 ; $p < 0.01$) and with GM130 (control, 0.672 ± 0.029 ; AKAP350 CTD, 0.617 ± 0.028 ; $p < 0.01$). Furthermore, analysis of Mander's coefficient (M) showed a diminished fraction of $R_{II\alpha}$ -PKA fluorescence associated with Golgi (control, 0.29 ± 0.05 ; AKAP350 CTD, 0.20 ± 0.04 ; $p < 0.01$) and centrosomal markers (control, 0.006 ± 0.001 ; AKAP350 CTD, 0.001 ± 0.001 ; $p < 0.01$) in cells expressing high levels of AKAP350 CTD-GFP. Consistently, morphometric estimation of percentage of $R_{II\alpha}$ -PKA fluorescence associated with centrosomes indicated that high levels of AKAP350 CTD-GFP induced a 60 % ($p < 0.01$) decrease in centrosomal PKA. Altogether, the results presented in this section indicate that AKAP350 participates in recruiting PKA to both the Golgi apparatus and the centrosomes in HepG2 cells.

Expression of AKAP350 CTD or a decrease in AKAP350 expression leads to an inhibition of the development of canalicular structures

AKAP350 is involved in maintaining the Golgi apparatus structure (Larocca et al., 2004), and in modulating microtubule dynamics (Takahashi et al., 2002; Keryer et al., 2003; Larocca et al., 2006; Rivero et al., 2009) in various epithelial cells. The Golgi apparatus sorts newly synthesized lipids and proteins into vesicles specifically targeted to each pole of the cell, while the microtubule cytoskeleton mediates their polarized traffic and insertion into the plasma membrane (Roelofsen et al., 1998; Schmoranzler and Simon, 2003). In order to analyze if AKAP350 was involved in the development of canalicular structures, we first assessed the effect of the displacement of AKAP350 from the Golgi apparatus and centrosomes on the formation of canalicular poles (Fig. 3), which were identified as F-actin and ABCC2 rich structures present in between two cells (chevrons). The analysis of phalloidin staining showed that expression of EGFP-AKAP350 CTD inhibited the formation of those structures (Fig. 3). In order to quantify our observations, we counted the EGFP-AKAP350 CTD transfected and non-transfected cells which had developed canalicular domains (Fig. 3, bars). The quantitative analysis indicated that EGFP-AKAP350 CTD expression induced a significant decrease of about 40 % in the number of polarized cells ($p < 0.05$).

In order to further support the hypothesis that AKAP350 participates in canalicular biogenesis, we analyzed the effect of specifically decreasing AKAP350 expression by RNA interference on the development of canalicular structures. Cells were transfected with RNA duplexes targeting AKAP350 nucleotides 9397–9417 (siRNA2), or 10917–10937 (siRNA1), or its scrambled control (siRNA SC), as we have described previously (Larocca et al., 2004). All of the experiments were performed 48 hours after transfection, when the cells transfected with siRNA1 or siRNA2 elicited a mean 75 % decrease in AKAP350 expression (Fig. 4A). In these conditions, total levels of actin and the canalicular protein ABCC2 remained unchanged (Fig. 4A). We identified canalicular poles as structures located between two cells, with intense specific staining for F-actin or for ABCC2, in images obtained by immunofluorescence confocal microscopy (Fig 4B, chevrons). The quantitative analysis of phalloidin and ABCC2 staining indicated that the number of canalicular structures per 100 cells was significantly reduced in siRNA1 transfected cells by 40 % ($p < 0.01$) and 25 %

($p < 0.05$), respectively. Similar results were obtained with siRNA2 (Fig 4B, bars). We also evaluated the effect of AKAP350 depletion on the formation of functional canaliculi, by analyzing the secretory ability of control or AKAP350 knock down live cells using the fluorophore GS-MF, which is actively transported into the bile canaliculi by ABCC2. The quantitative analysis of GS-MF positive canaliculi (Fig. 4C, chevrons) indicated a significant decreased number of secretory apical structures in cells transfected with siRNA1 and siRNA2 (siRNA1 -45 %, siRNA2 -40 %, $p < 0.05$). Thus, in accordance with the immunofluorescence data, the decrease in AKAP350 expression induced a significant decrease in the formation of functional canalicular structures.

AKAP350 modulates the canalicular localization of ABCB1

Among the canalicular proteins, the ABC transporters comprise a group of proteins with essential roles in bile secretion, which include: the bile salt export pump (BSEP) and ABCC2 for organic anions transport, and ABCB1 for organic cations. To study if AKAP350 was also necessary for polarized expression of canalicular proteins in cells which developed canalicular structures we evaluated the effect of AKAP350 knockdown on ABCC2 and ABCB1 localization (Fig. 5). HepG2 cells were transfected with siRNA1 (AKAP350 KD) or its scrambled control (Control). Analysis of ABCC2 and ABCB1 levels by immunoblotting indicated that AKAP350 depletion did not affect the global expression of these proteins (Fig. 5A). Confirming the data shown in Fig. 4B, quantitative analysis of canalicular poles using ABCB1 and phalloidin staining indicated that AKAP350 knock down inhibited the formation of ABCB1 positive canalicular structures (data not shown). To analyze the canalicular localization of ABCB1 and ABCC2, we first performed a colocalization analysis, as described in Materials and Methods. The results showed a decrease in ABCB1/actin (Fig. 5B) and ABCC2/actin (Fig. 5C) colocalization in AKAP350 KD cells. Because the colocalization test evaluates the whole population of cells, and the number of canaliculi developed by AKAP350 KD cells is lower than that of control cells, we performed a morphometric study considering only the polarized cells to analyze if the decreases in ABCB1 and ABCC2 colocalization with canalicular actin were due to a defect in ABCB1 and/or ABCC2 canalicular localization in the polarized AKAP350 KD cells. Our results showed that AKAP350 depletion led to decreased canalicular ABCB1 levels (Fig. 5B), without changing canalicular ABCC2 expression in polarized cells (Fig. 5C). It is noteworthy that we did not find any evidence of basolateral localization of ABCB1, indicating that its decreased canalicular expression was not due to a mis-targeting or diffusion of ABCB1 to the basolateral membrane. Thus, the decreased ABCC2 colocalization with canalicular actin was probably secondary to the decreased number of polarized cells in the AKAP350 KD group with respect to the controls (Fig. 4B). On the other hand, AKAP350 KD cells which did polarize had reduced canalicular expression of ABCB1, indicating that the factors determining ABCB1 polarized localization are sensitive to changes in AKAP350 expression even in cells which developed canalicular structures.

AKAP350 depletion alters canalicular actin nucleation after cytochalasin D treatment

Actin cytoskeleton dynamics are involved in different events related to bile canaliculi formation (Roma *et al.*, 2000; Tuma *et al.*, 2002). Thus, we employed an experimental model of F-actin disruption to study canalicular actin nucleation in cells that had reduced AKAP350 expression. Control and AKAP350 KD cells were treated with cytochalasin D, in conditions that completely disorganize the canalicular F-actin cytoskeleton, but did not delocalize canalicular ABCC2 (Fig. 6). Following the treatment, the cells were washed with PBS, and re-incubated with cytochalasin D-free media. After 60 minutes of recovery, both control and AKAP350 KD cells showed a similar general restoration of F-actin cytoskeleton (Fig. 6A). Nevertheless, it was noteworthy that canalicular actin was less re-organized in AKAP350 KD cells. The morphometric analysis of the polarized cells -identified by ABCC2

staining- indicated that the reduction in AKAP350 expression inhibited F-actin organization at the canaliculi by about 40% ($p < 0.01$, Fig. 6B). Since it was evident that after 1 h recovery from cytochalasin D treatment there was some disorganization in ABCC2 staining in AKAP350 KD cells, we quantified the percentage of ABCC2 staining located at the canalicular structures in the different groups of cells (Fig. 6C). The morphometric analysis of three independent experiments indicated that, even though ABCC2 canalicular localization was not affected by the disruption of actin cytoskeleton either in control or in AKAP350 KD cells, after 60 minutes of recovery, AKAP350 KD cells showed a significant decrease in canalicular ABCC2 that was not observed in control cells. Considering that the decrease in AKAP350 levels did not affect ABCC2 expression (Fig. 4A, 5A) or canalicular localization (Fig. 5C, 6C) in basal conditions, these results could indicate a defect in ABCC2 canalicular retention by the repolymerizing actin cytoskeleton in AKAP350-depleted cells.

Golgi nucleated-microtubules are necessary for proper canalicular biogenesis

Even though centrosomes are considered the main microtubule organizing center (MTOC), the Golgi apparatus also behaves as an MTOC, findings that were first described in hepatic cells (Chabin-Brion et al., 2001). AKAP350 participates in microtubule organization at the Golgi apparatus by recruiting the γ -TURC proteins, which are directly responsible for microtubule nucleation (Rivero et al., 2009). Previous studies demonstrated that Golgi-derived microtubules are necessary for establishing the cortical actin cytoskeleton at the leading edge and vectorial migration of epithelial migrating cells (Miller et al., 2009). Thus, we next analyzed if Golgi apparatus-derived microtubules were necessary for canalicular actin nucleation and apical polarity development in HepG2 cells. Centrosome and Golgi dependent microtubule nucleation share effectors, such as AKAP350 and γ -TURC proteins (Rivero et al., 2009). In contrast, CLASP1 and CLASP2 proteins specifically stabilize Golgi-derived microtubules (Miller et al., 2009). In order to analyze if microtubule nucleation at the Golgi apparatus was involved in the mechanism by which AKAP350 participates in apical polarity development in HepG2 cells, we specifically inhibited the formation of Golgi nucleated microtubules by decreasing expression of CLASP1 and CLASP2, as previously described by others (Miller et al., 2009). We first analyzed the development of apical polarity in CLASP1/2 depleted cells (CLASP KD) (Fig. 7). The results from four independent experiments indicate that the decrease in CLASP1/2 expression led to a 40 % inhibition in the development of canalicular structures ($p < 0.05$, Fig. 7B).

Considering that AKAP350 deficient cells were defective in canalicular nucleation of actin, we next analyzed if Golgi-derived microtubules were necessary for proper nucleation of canalicular actin. The results from three independent experiments indicated that the decrease in CLASP1/2 expression by RNA interference inhibited by about 35% canalicular F-actin recovery after cytochalasin D treatment ($p < 0.05$, Fig. 8).

DISCUSSION

AKAP350 is a scaffolding protein that organizes protein complexes at the Golgi apparatus and centrosomes in epithelial derived cell lines. Among the proteins nucleated by AKAP350, PKA, CIP4, GCP2/3 and Ran modulate different functions associated with cell polarity, such as polarized traffic of lipids and proteins and modulation of microfilament and microtubule cytoskeleton. In fact, AKAP350 has been implicated in microtubule nucleation at the Golgi apparatus, and in the modulation of the structure of this organelle. Considering that the Golgi apparatus and the microtubules participate in the targeted delivery of apical and basolateral membrane components in epithelial cells, our aim was to evaluate the role of AKAP350 in the establishment of apical cell polarity in the hepatocyte derived HepG2 cells. Our main findings reveal that AKAP350 is involved in the development of bile canaliculi-

like structures in HepG2 cells. In addition, we observed that decreased expression of AKAP350 led to defects in both nucleation of canalicular actin cytoskeleton and polarized expression of canalicular proteins. Finally, we demonstrated that canalicular biogenesis and canalicular nucleation of F-actin depends on microtubule nucleation by the Golgi apparatus.

The apical cytoskeleton is implicated in the development and maintenance of luminal polarity in epithelial cells (Tuma et al., 2002; Mege et al., 2006; Weisz and Rodriguez-Boulant, 2009). In hepatocytes, the pericanalicular actin cytoskeleton regulates different processes associated with bile secretion, such as vesicle insertion and endocytosis at the plasma membrane, and bile canaliculus contractions. Indeed, alterations in actin cytoskeleton lead to impairments in bile secretion (Elias et al., 1980; Rost et al., 1999). Our studies reveal that inhibition of the development of canalicular structures in AKAP350 knockdown cells is associated with a defective apical F-actin organization at the canalicular poles. These observations are relevant not only to the mechanism leading to AKAP350-dependent canalicular biogenesis, but also for the normal function of the canaliculi once they are developed. In this regard, we showed that during recovery of the actin cytoskeleton after cytochalasin D treatment, control cells maintain their canalicular levels of ABCC2, while AKAP350 KD cells show a partial redistribution of the protein to the cytosol. Among the proteins associated with canalicular F-actin, the ezrin-radixin-moesin proteins are implicated in apical retention of proteins, including ABCC2 (Kikuchi et al., 2002). Our results could indicate that F-actin reconstituted in those HepG2 cells with decreased expression of AKAP350 allows endocytosis of canalicular MRP2, but is defective in retaining the protein at the canalicular structures, possibly due to an abnormal organization of its associated proteins.

Our results showed that AKAP350 colocalizes with PKA-R_{IIα} at subapical centrosomes and the Golgi apparatus, and that delocalization of AKAP350 leads to the release of PKA from these organelles, thus confirming that AKAP350 is, at least partially, responsible for recruiting PKA to these subcellular compartments in HepG2 cells. PKA is one of the signalling proteins which regulates the establishment and maintenance of epithelial polarity in response to intracellular and extracellular signals. Activation of PKA leads to accelerated formation of Golgi apparatus-derived vesicles (Muñiz et al., 1997) and increased transcytotic traffic from the basolateral to the apical membrane in MDCK cells (Hansen and Casanova, 1994). In hepatocytes, PKA stimulates canalicular biogenesis (Zegers and Hoekstra, 1997) and mediates the regulated insertion of vesicles containing canalicular proteins into the canalicular membrane (Gatmaitan et al., 1997; Kipp and Arias, 2000; Gradilone et al., 2003). Van Ijzendoorn and colleagues demonstrated that PKA-R_{II} interaction with its anchoring proteins is required for efficient traffic of ABCB1 from the Golgi apparatus to the canalicular membrane, but not for canalicular biogenesis in basal conditions (Wojtal et al., 2006). Our results indicated that AKAP350-depleted cells which developed canalicular poles had a defect in the polarized expression of ABCB1, implicating AKAP350 participation in mechanisms leading to canalicular trafficking of ABCB1 independent of its involvement in the formation of canalicular poles. Thus, an appealing hypothesis is that AKAP350 recruitment of PKA to specific centrosomal and/or Golgi protein complexes modulates ABCB1 trafficking towards the canaliculus, while a different mechanism mediates the AKAP350 role in canalicular development in basal conditions.

An intimate connection has been established between focal nucleation of actin at the plasma membrane and microtubule cytoskeleton dynamics (Li and Gundersen, 2008). AKAP350 is implicated in microtubule nucleation at the centrosomes and the Golgi apparatus (Takahashi et al., 2002; Keryer et al., 2003; Rivero et al., 2009), both of which are responsible for nucleating microtubules in hepatic cells (Chabin-Brion et al., 2001). In previous studies we found that a decrease in AKAP350 expression by RNA interference did not influence

centrosomal nucleation of microtubules. These findings could be related to the fact that reduction of centrosomal AKAP350 is not as effective as reduction of Golgi apparatus AKAP350 (Larocca et al., 2006). Similarly, Rios and colleagues observed that AKAP350 depletion by RNA interference did not impair the formation of centrosomal derived microtubules, but did compromise microtubule nucleation by the Golgi apparatus (Rivero et al., 2009). It was recently demonstrated that nucleation of microtubules at the Golgi apparatus is necessary for establishing the cortical actin cytoskeleton at the leading edge, polarized vesicle trafficking, and vectorial migration of epithelial cells (Miller et al., 2009). Indeed, the impairment in microtubule nucleation at the Golgi apparatus secondary to the decrease in AKAP350 expression leads to defective cell migration in these cells (Rivero et al., 2009). Even though the pathways associated with establishment of polarity may vary depending on the cell and polarity type, it appeared reasonable to hypothesize that microtubule nucleation at the Golgi apparatus was necessary for apical polarity development in HepG2 cells. Our results demonstrated that inhibition of the formation of Golgi-derived microtubules by CLASP1/2 depletion induced a diminished number of polarized cells and impaired nucleation of canalicular actin. Thus, these findings indicate that Golgi-derived microtubules are necessary for normal formation of canalicular structures and suggest a role for AKAP350 in the development of apical polarity in HepG2 cells related to its function in facilitating microtubule nucleation at the Golgi apparatus.

In summary, we have demonstrated that AKAP350 has a major role in the development of canalicular structures: loss of AKAP350 function leads to decreased formation of canalicular poles and impaired canalicular actin cytoskeleton nucleation. The inhibition in the formation of Golgi derived microtubules by a different mechanism also inhibits the formation of canalicular structures and canalicular F-actin nucleation. On the other hand, the decrease in AKAP350 expression leads to defective canalicular localization of ABCB1 in polarized cells, a process which depends on PKA interaction with its anchoring proteins. Our data support the hypothesis that, by scaffolding proteins involved in microtubule nucleation at the Golgi apparatus and regulatory proteins such as PKA, AKAP350 integrates pathways regulating the structural basis of bile secretion.

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Figure 1.

PKA colocalizes with AKAP350 at the Golgi apparatus and subapical centrosomes in HepG2 cells. Cells were grown for 48 hours, fixed and stained using anti-AKAP350A (A–C), anti GM-130 (A), anti γ -tubulin (B) or anti-R_{IIα} PKA (C) antibodies, and phalloidin (A, C) to identify canalicular structures (chevrons). The analysis by immunofluorescence microscopy shows that AKAP350 localized at the Golgi apparatus (A) as well as at the centrosomes (B, arrows). AKAP350 colocalized with PKA-R_{IIα} both at the Golgi apparatus (C, arrowheads) and at subcanalicular centrosomes (C, arrows). Bar, 5 μ m.

Figure 2.

AKAP350 displacement from the centrosomes and the Golgi apparatus leads to PKA release from these organelles. HepG2 cells were transiently transfected with pEGFP-AKAP350 CTD, which contains AKAP350 centrosomal and Golgi targeting domains fused to EGFP. After 16 h, cells were fixed and dual stained with anti-AKAP350 (A, B) or anti-R_{II} α -PKA (C, D) and phalloidin (A), anti GOLPH4 (B), anti γ -tubulin (C) or anti GM130 (D). Immunofluorescence images were obtained by confocal microscopy. The first column shows pEGFP-AKAP350 CTD transfected cells. Stars indicate cells expressing high levels of AKAP350 CTD-GFP (AKAP350 CTD). Arrows indicate centrosomes, and arrowheads Golgi apparatus. Bar charts show the percentage of PKA fluorescence associated with the centrosomes or the Golgi apparatus in control and AKAP350 CTD cells, determined as described in Material and methods. Results are representative of three independent experiments. Bar, 5 μ m. * $p < 0.01$.

Figure 3.

AKAP350 delocalization inhibits the formation of canalicular structures. HepG2 cells were transiently transfected with pEGFP-AKAP350 CTD (AKAP350 CTD-GFP) or with the empty vector pEGFP (EGFP). After 72 hours, cells were fixed and dual stained with phalloidin and anti-ABCC2, to identify canalicular structures (chevrons). Images were obtained using confocal microscopy. Stars indicate the position of cells expressing high levels of AKAP350 CTD-GFP or EGFP. Quantitative analysis of the images was performed to determine the percentage of cells which developed canalicular structures (polarized cells). The bars represent the percentage of polarized cells corresponding to cells that did not express (Control) or expressed high levels of AKAP350 CTD-GFP (AKAP350-CTD). EGFP expression alone did not alter the percentage of polarized cells (control: 47% ± 4%, EGFP: 51% ± 1%). Results are means ± s.e.m. of three experiments. Bar: 5 μ m. * $p < 0.05$.

Figure 4.

AKAP350 knock down inhibits the normal development of canalicular structures. HepG2 cells were transfected with siRNA1 or siRNA2 (AKAP350 KD), specifically targeted to AKAP350 mRNA, or with the siRNA SC (control), synthesized using siRNA1 scrambled sequence, and allowed to grow for 48 hours. A: AKAP350, ABCC2 and β -actin expression were analyzed by western blot, in control and AKAP350 KD cells. α -tubulin was used as loading control. B: Canalicular structures were identified as actin or ABCC2 enriched structures present in between two or more cells (chevrons). Control and AKAP350 KD cells were fixed and stained with phalloidin (first row), anti-ABCC2 antibody (second row) and DAPI, and images were obtained by confocal microscopy. The third row shows the merged images of phalloidin (red), ABCC2 (green) and DAPI (blue) staining. The number of bile canaliculi stained with phalloidin (bars, upper chart) or ABCC2 (bars, lower chart) relative

to the number of nuclei was quantified in at least 500 cells in each experiment. Results are means $\pm$ s.e.m. of five (siRNA1) or three (siRNA2) experiments. C: Functional canaliculi structures were recognized as vacuoles present between two or more cells which could actively accumulate the ABCC2 substrate GS-MF (chevrons). Control and AKAP350 KD cells were incubated with CMFDA for 10 min. Accumulation of CMFDA metabolite GS-MF in canaliculi lumens was analyzed by fluorescence microscopy in at least 250 cells in each experiment. The bars represent the number of canaliculi which accumulated GS-MF every 100 cells. Results are means $\pm$ s.e.m. of three experiments. Bar: 5 μ m. * p <0.05; ** p <0.01.

Figure 5.

AKAP350 expression is necessary for canalicular localization of ABCB1. HepG2 cells transfected with siRNA SC (Control) or siRNA1 (AKAP350 KD) were grown for 48 hours and evaluated for ABCB1 and ABCC2 expression. A: Total expression of AKAP350, ABCB1 and ABCC2 was analyzed by immunoblotting. α -tubulin was used as loading control. B, C: Cells were fixed and dual stained with anti-ABCB1 or anti-ABCC2 antibodies and phalloidin. Immunofluorescence images were obtained by confocal microscopy and analyzed as described in Materials and methods. Colocalization studies between the ABCC2 or ABCB1 and the actin channels were performed using an Image J colocalization plug-in. The first chart shows the Pearson's coefficient of correlation (Rr) between either ABCB1 or ABCC2 and canalicular actin signals, which is a parameter that estimates the canalicular localization of the proteins in the whole population of each group of cells. In order to quantify ABCB1 and ABCC2 canalicular localization in polarized cells, we determined the canalicular and the total intensity of cell fluorescence in at least 50 polarized cells, and calculated canalicular fluorescence as a percentage of total cell fluorescence to estimate the percentage of ABCB1 (B) or ABCC2 (C) present at the canaliculus in these cells (second

chart). Results are representative of three independent experiments. Chevrons indicate canalicular structures. Bar: 10 μ m. * $p < 0.01$

Figure 6.

Decrease of AKAP350 expression inhibits canalicular actin nucleation after cytochalasin treatment. HepG2 cells were transfected with siRNA SC (Control) or siRNA1 (AKAP350 KD) and allowed to grow for 48 hours. Control and AKAP350 KD cells were treated with 2 μ M cytochalasin D for 60 minutes, conditions that induce the depolymerization of canalicular actin cytoskeleton (Cytochalasin). Cells were washed 3 times with PBS and incubated for 60 minutes in cytochalasin D free media (Recovery). A: Control and AKAP350 KD cells were fixed and stained with phalloidin, anti-ABCC2 antibody and DAPI. Images were obtained using confocal microscopy. ABCC2 staining was used to identify canalicular structures in Cytochalasin and Recovery cells. The merged images show actin (red), ABCC2 (green) and nuclei (blue) staining. Chevrons indicate F-actin or ABCC2 at the canaliculus. B, C: In order to quantify canalicular actin nucleation, and ABCC2 canaliculus localization, we determined the canaliculus and the total intensity of cell fluorescence in at least 50 polarized cells, and calculated canaliculus fluorescence as a percentage of total cell fluorescence to estimate the percentage of the total F-actin (B) or ABCC2 (C) present at the canaliculus (canaliculus actin (%)) and canaliculus ABCC2 (%), respectively). Results are representative of four (B) or three (C) independent experiments. Bar: 10 μ m. * p <0.01.

Figure 7.

The decrease in CLASP1/2 expression impairs the development of apical polarity in HepG2 cells. Cells were transfected with RNA duplexes specifically targeted to CLASP1 and CLASP2 (CLASP KD) or with the siRNA SC (control), and allowed to grow for 48 hours. A: CLASP1 and CLASP2 expression was analyzed by immunoblotting in control and CLASP KD cells. α -tubulin was used as loading control. B: Control and CLASP KD cells were fixed and stained with phalloidin, anti-ABCC2 and DAPI and analyzed by confocal microscopy. The number of canalicular structures stained with phalloidin (chevrons) relative to the number of nuclei stained with DAPI was quantified in at least 500 cells in each experiment (bars). Results are means $\pm$ s.e.m. of four independent experiments Bar: 5 μ m. * $p < 0.05$.

Figure 8.

Decrease of CLASP1/2 expression inhibits canalicular actin nucleation after cytochalasin D treatment. HepG2 cells were transfected with siRNA SC (Control) or the mix of CLASP1 and CLASP2 targeted siRNAs (CLASP KD) and allowed to grow for 48 hours. Control and CLASP KD cells were treated with 2 μ M cytochalasin D for 60 minutes to induce F-actin disruption (Cytochalasin). Cells were washed 3 times with PBS, and incubated for 60 minutes in cytochalasin D free media (Recovery). A: Control and CLASP KD cells were fixed and stained with phalloidin and anti-ABCC2, and the images were obtained using confocal microscopy. B: In order to quantify canalicular actin nucleation, we determined the canalicular and the total intensity of cell fluorescence in polarized cells in, at least, 6 different fields, and calculated canalicular fluorescence as a percentage of total cell fluorescence to estimate the percentage of the total F-actin present at the canaliculus (canalicular actin (%), bars). Results are representative of three independent experiments. Chevrons indicate canalicular structures. Bar: 10 μ m. * p <0.05.