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ATP-binding cassette transporter isoform C2 localizes to the apical plasma membrane via interactions with scaffolding protein

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ATP-binding cassette transporter isoform C2 (ABCC2) localizes to the apical plasma membrane in polarized cells. Apical localization of ABCC2 in hepatocytes plays an important role in biliary excretion of endobiotics and xenobiotics, but the mechanism by which ABCC2 localizes to the apical membrane has not been conclusively elucidated. Here, we investigate the role of scaffolding proteins on ABCC2 localization with a focus on the function of PDZK1 (post-synaptic density 95/disk large/zonula occludens-1 domain containing 1) in regulating ABCC2 localization. The C-terminal 77 residues of ABCC2 were used to probe interacting proteins from HepG2 cells. Protein mass fingerprinting identified PDZK1 as a major interacting protein. PDZK1 associated with the plasma membrane, most likely at the apical vacuoles of HepG2 cells. Affinity pull-down assays confirmed that the C-terminal NSTKF of ABCC2 bound to the fourth PDZ domain of PDZK1. Removal of this PDZ-binding motif significantly reduced the normal apical localization of ABCC2. In HepG2 cells, overexpression of this fourth domain overcame endogenous PDZK1 and reduced the ABCC2 localization at the apical membrane with a reciprocal increase of intracellular accumulation of mislocalized ABCC2. These results suggest a possible role for an interaction between ABCC2 and PDZK1 in apical localization of ABCC2 in hepatocytes.

Keywords: ATP-binding cassette transporter/apical plasma membrane/bile canaliculus/PDZK1/polarized cell.

Abbreviations: ABCC2, ATP-binding cassette transporter isoform C2; CTD, C-terminal domain; ERM, ezrin-radixin-moesin; GST, glutathione S-transferase; NHERF, Na^+/H^+ exchanger regulating factor; PCR, polymerase chain reaction; PDZ, post-synaptic density 95/disk large/zonula occludens-1; PDZK1, PDZ domain-containing kidney protein 1.

Hepatocytes are polarized cells that are characterized by their ability to differentiate morphologically and functionally discrete domains in plasma membranes. The canalicular membranes of adjacent hepatocytes form the bile canaliculi and occupy the apical domain, whereas the sinusoidal membranes of individual hepatocytes encompass the Disse space and occupy the basolateral domain (*1*). Formation and maintenance of a polarized distribution of proteins in these two domains are crucial for the ability of hepatocytes to execute various hepatic activities, including vectorial transport of metabolites; therefore, polarity of hepatocytes is critical to organismal homeostasis. Most transporter proteins trafficked to the plasma membrane are sorted to different domains. ATP-binding cassette (ABC) transporters comprise a large family of multipass transmembrane proteins and are divided into seven subfamilies from A to G (*2*). ABC transporters are present in membranes of various organelles, where they play crucial roles in vectorial transport of a variety of compounds. The human ABC transporter subfamily C (ABCC) includes 13 isoforms of cellular export pumps and exhibits a polarized distribution in the plasma membrane (*3*). One ABCC family member, ABCC2, was originally identified as an organic anion transporter located in the canalicular membrane of hepatocytes that transports glucuronidated bilirubin from the liver into the bile (*4*). The bile canaliculi function as an important route for systemic elimination of potentially toxic endobiotics and xenobiotics, and this elimination is mediated by canalicular efflux transporters, including ABCC2.

Localization at the apical plasma membrane is presumably required for the physiological functions of ABCC2 (*4*). Several studies have attempted to establish the involvement of distinct signals of ABCC2 in control of apical localization by analysing various ABCC2–ABCC1 chimeric proteins (*5*, *6*) or series of step-wise deletion constructs of ABCC2 (*7*). However, a shortly defined amino acid motif responsible for the apical localization of ABCC2 in polarized epithelial cells has not been conclusively established. It is considered that the apical-targeting signal of ABCC2 is unlikely to be a short amino acid sequence, but rather appears to become functional in combination with several motifs from different regions of ABCC2 that come together only in the native conformation of the intact protein. The C-terminal cytoplasmic region of ABCC2 has been proposed to contain such additional regulatory elements for apical localization (*3*, *4*).

In general, a complex array of protein–protein interactions governs sorting of proteins bound for the plasma membranes, internalization of delivered proteins into destination membranes and formation of certain macromolecular complex of installed proteins (*8*). Adapter proteins are known to link cell surface membrane proteins to cellular components, including the F-actin cytoskeleton. Radixin is a member of the ezrin–radixin–moesin (ERM) family proteins and apparently required for the apical localization of ABCC2 (*9*). Post-synaptic density 95/disks large/zonula occludens-1 (PDZ) domains are a large family of protein binding modules and have been identified in a wide variety of proteins (*10*, *11*). PDZ-containing kidney protein 1 (PDZK1) and Na^+/H^+ exchanger regulating factors (NHERFs) are multivalent PDZ proteins and have recently been proposed to act as adapters for transporters on the plasma membrane (*12–14*). These multivalent PDZ proteins tether interacting proteins to the plasma membrane through interactions with a protein network consisting of the F-actin cytoskeleton and other adapter proteins, such as ERM proteins. The C-terminal region of ABCC2 may contain a binding motif for PDZ domain proteins (*4*). However, the identity of the PDZ adapter protein(s) interacting with ABCC2 has not been conclusively established.

A possible interaction between ABCC2 and PDZK1 was shown in yeast two-hybrid experiments (*15*), though PDZK1 knock-out mice have normal apical localization of ABCC2 in kidney and liver (*16*). In addition, the role of the C-terminal PDZ-binding motif in control of the apical expression of ABCC2 remains controversial. A previous report showed that deletion of this PDZ-binding motif resulted in altered localization of ABCC2 to the basolateral membrane of polarized MDCK cells (*17*), whereas other subsequent studies have shown that this motif was not required for the apical localization of ABCC2 and could be deleted without loss of apical localization (*5–7*, *18*). It is noteworthy that these studies analysed recombinant proteins expressed in model cell lines, and that variations in the experimental design, such as positioning of epitope tags in recombinant proteins, the choice of cell lines and the amount of expression plasmid brought into cells, influenced the intracellular localization of the expressed proteins. Nies and co-workers (*7*) pointed out that the inconsistent results of the preceding reports may result from different experimental setups that mask the PDZ-interacting motif by fusing GFP to the C-terminus of ABCC2 and express the recombinant proteins in MDCK cells. This canine cell line has a higher tendency for altered sorting of exogenous proteins of unspecified origin than other polarized cells, such as porcine LLC-PK1 cells (*19*) and human HepG2 cells (*7*). In addition, these conflicting results may stem from the transfection conditions; specifically, a relatively large amount of expression plasmids may elicit overproduction of recombinant proteins and cause ectopic targeting of the recombinant protein to different subcellular compartments. In particular, ER-associated reticular distribution can frequently arise from overproduction of membrane proteins. In a similar situation, enhanced expression of the Golgi-directed ABCB6 protein resulted in unexpected accumulation in the ER (*20*).

To overcome these unfavourable effects, we prepared human ABCC2 fused with FLAG-tags at the N-terminus instead of the C-terminal GFP-fusions, used relatively small amounts of expression plasmids in transient transfection of HepG2 cells to avoid overproduction of recombinant proteins, and determined the percentage of cells displaying distinct localization pattern by excluding cells that exhibited dense staining of the FLAG-ABCC2. Here, we report the role of PDZK1 in the apical localization of ABCC2 with a focus on the roles played by the fourth PDZ domain of PDZK1 in regulating the apical localization of ABCC2, and the importance of the C-terminal PDZ-binding motif of ABCC2 in regulating its own apical localization.

Materials and Methods

Expression of ABCC2 and PDZK1 in HepG2 cells

ABCC2 cDNA was isolated from human liver Quick-Clone cDNA (Promega, Madison, WI, USA) by polymerase chain reaction (PCR) using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) as described in the supplier's instruction manual. The primers used for amplification of human ABCC2 cDNA (NM000392) are as follows: C2-F, 5′-CCCAAGCTTGCGGCCGCCATGCTGGAGAAG TTCTGCAAC-3′ (the recognition sites of HindIII and NotI are underlined); C2-R, 5′-GGAAGATCTCTAGAATTTTGTGCTGT TCACATTC-3′ (the BglII site is underlined). The amplified DNA fragment containing the entire coding sequence was treated with NotI plus BglII and ligated into p3×FLAG-CMV-9 (Sigma, St Louis, MO, USA). The preprotrypsin signal peptide preceded the three consecutive FLAG sequences and conferred accurate topology of the recombinant protein in the plasma membrane. The resultant expression plasmid, pCMV9-ABCC2, produced an N-terminally tagged version of the ABCC2 protein (FLAG-ABCC2) in cultured mammalian cells. C-terminal deletions of FLAG-ABCC2 were created using the QuickChange site-directed mutagenesis kit (Stratagene) according to the supplier's protocol.

Human PDZK1 (NM002614) cDNA was amplified by PCR as described above. The primers used for amplification were as follows: PDZK1-F, 5′-ACGCGTCGACCATGACCTCCACCTTCAA CCCC-3′ (the SalI recognition site is underlined); PDZK1-R, 5′-CCGCTCGAGTCACATCTCTGTATCTTCAGAATTG-3′ (the XhoI recognition site is underlined). The amplified PCR products were treated with SalI plus XhoI and then ligated into pCMV-HA (Clontech, Mountain View, CA, USA) to generate pCMV-PDZK1, which produced an N-terminally tagged version of the PDZK1 protein (HA-PDZK1). The nucleotide sequence of the amplified cDNA fragments were confirmed by dideoxy sequencing with a Prism 3100 Avant sequencer (Applied Biosystems, Foster City, CA, USA).

HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum under an atmosphere of 5% CO_2 at 37°C. Transient transfection experiments were carried out essentially as described previously (*20*). Briefly, 2.0×10^5 cells were seeded into a 3.5-cm dish and cultured for 24 h. Cells were transfected using FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the supplier's protocol. Transfected cells were harvested, disrupted in SDS–PAGE sample buffer by sonication and centrifuged at 18,000*g* for 5 min. The clarified supernatant was subjected to immunoblot analysis either with anti-HA monoclonal antibodies (Covance, Princeton, NJ, USA) or anti-FLAG monoclonal antibodies (Sigma). Immunoreactive protein bands were probed with peroxidase-conjugated goat anti-mouse IgG (Covance) and visualized using ECL reagent (Amersham Biosciences, Piscataway, NJ, USA) with an LAS1000 image analyzer (Fuji, Tokyo, Japan).

Analysis of binding proteins for the ABCC2 C-terminal tail by affinity capture purification

Generation of glutathione *S*-transferase (GST) fusion proteins was carried out by PCR using PfuTurbo DNA polymerase with primers containing in-frame restriction enzyme sites. The amplified DNA fragment, containing the coding sequence for the ABCC2 C-terminal domain (CTD) comprising amino acids 1469–1545 (C-terminal 77 amino acids), was fused at the SmaI/XhoI site in pGEX-2T (Amersham-Pharmacia, Piscataway, NJ, USA) to produce a GST-CTD77 fusion protein. GST and the GST-CTD77 were separately expressed in *Escherichia coli* BL21 strain and affinity purified with glutathione–agarose columns. Cell extracts were prepared essentially as described (*21*) with minor modifications. HepG2 cells were homogenized in extraction buffer containing 120 mM NaCl, 20 mM HEPES-KOH (pH 7.5), 1% Triton X-100, 5 mM EDTA, 0.5 mM dithiothreitol and Complete Protease Inhibitor Cocktail (Roche Diagnostics). Lysate was collected after centrifugation of the homogenate at 18,000*g* for 30 min at 4°C and mixed with glutathione–agarose beads (Sigma). After gentle rotation for 30 min at 4°C, the precleared lysate was collected after centrifugation at 18,000*g* for 5 min at 4°C. The resultant supernatant was incubated with either GST or GST-CTD77 fusion protein which had been bound to glutathione–agarose. After gentle rotation for 1 h at 4°C, the beads were sequentially washed once with extraction buffer, twice with high-salt washing buffer (extraction buffer supplemented with 500 mM NaCl) and three times with washing buffer (extraction buffer without Triton X-100). Prior to analysis by SDS–PAGE, the bound proteins were eluted with Laemmli's sample buffer. Protein bands that appeared to exhibit specific interaction with GST-CTD77 were subjected to in-gel digestion with trypsin and subsequent MALDI/TOF mass spectrometry as described previously (*22*).

Affinity pull-down experiments

Site-directed mutagenesis was carried out using the QuickChang kit (Stratagene) to introduce successive alanine scanning substitutions into GST-CTD77. GST alone and the series of mutated GST-CTD77 fusion proteins were separately expressed in *E. coli* BL21 strain and affinity purified using glutathione–agarose beads. Generation of HA-tagged protein was carried out by PfuTurbo DNA polymerase with primers containing in-frame restriction enzyme sites. Amplified DNA fragments containing individual PDZ domains were treated with SalI plus XhoI and then ligated into pCMV-HA. These N-terminally tagged versions of PDZ domains were separately expressed in HepG2 cells. The affinity pull-down experiments were carried out according to procedures that are essentially identical to those used in the analysis of the CTD77-binding proteins. Bound proteins were eluted with Laemmli's sample buffer and subjected to SDS–PAGE followed by immunoblot detection with anti-HA monoclonal antibodies.

Indirect immunofluorescence microscopy

Cells grown on 18 mm × 18 mm cover glasses were fixed using 4% (w/v) paraformaldehyde in phosphate-buffered saline for 15 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 15 min and then subjected to blocking in phosphate-buffered saline containing 1% (w/v) bovine serum albumin for 30 min. To detect epitope-tagged proteins by immunostaining, the processed cells were incubated sequentially with either anti-HA monoclonal antibodies (1 : 1,000 dilution) or anti-FLAG monoclonal antibodies (1 : 500 dilution) for 1 h and Alexa Fluor 488-conjugated anti-mouse IgG (1 : 1,000 dilution) for 1 h. Rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) was used to stain the apical vacuoles formed between adjacent HepG2 cells. Anti-PDZK1 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used for immunostaining of endogenous PDZK1. The cells were mounted on slide glasses with Vectashield anti-fade mounting medium (Vector Laboratories, Burlingame, CA, USA), and immunofluorescence observation was carried out essentially as described previously (*23*).

Results

Apical localization of ABCC2 in polarized HepG2 cells

Hepatocarcinoma-derived HepG2 cells are not normal hepatocytes, but they are functionally and structurally polarized like hepatocytes in liver tissues. A significant number of HepG2 cells develop polarity over time in monolayer culture, resulting in the formation of round and sealed vacuoles among the plasma membrane of two adjacent cells (Fig. 1A). Membranes of the bile canaliculi of hepatocytes and the vacuoles of HepG2 cells correspond to the apical domain of the plasma membrane (*24*). Actin filaments are present in abundance just beneath the plasma membrane and particularly enriched in the apical domain; therefore, the outlines of this apical vacuoles were readily visualized under epifluorescence microscopy using rhodamine-conjugated phalloidin, a sensitive stain for F-actin (*25*). The apical vacuoles were particularly visible as a single punctuated staining between adjacent cells (Fig. 1).

To examine the localization of transiently expressed ABCC2, we constructed a tagged version of ABCC2 by fusing three consecutive FLAG-tags to the N-terminus of ABCC2 (FLAG-ABCC2). The C-terminus of this ABCC2 fusion is free to interact with other proteins necessary for apical localization. An additional preprotrypsin signal peptide was placed just in front of the FLAG-tags in order to allow the N-terminus of the FLAG-ABCC2 fusion protein to translocate into the ER lumen and eventually to be located on the extracellular face of the plasma membrane. As shown in Fig. 1, the fluorescence from FLAG-ABCC2 gave a single large-punctuated signal between neighbouring cells and was completely merged with that from the apical vacuoles decorated with rhodamine-conjugated phalloidin (Fig. 1B and D, arrow heads). On the contrary, in cells without any hallmarks of polarization, FLAG-ABCC2 appeared to be associated with intracellular vesicular structures (Fig. 1B and E, asterisks). ABCC1 and ABCC2 are closely related members of the C family of ABC transporters that are sorted to discrete domains in polarized epithelial cells despite their homology (*3*). An ABCC1 FLAG-tagged fusion protein (FLAG-ABCC1) expressed in HepG2 cells was distributed to regions of the cell perimeters other than the apical domain (Fig. 1C and F). Extracellular staining of FLAG-ABCC1 was observed in non-permeabilized cells, suggesting basolateral localization of FLAG-ABCC1 in the polarized HepG2 cells (data not shown). These results indicated that fusion of ABCC2 or ABCC1 to the FLAG tag along with the preceding signal peptide results in localization of these fusion proteins that was similar to their endogenous counterparts and further suggestsed that HepG2 cells contained the cellular machinery necessary for the correct localization of ABCC2 to the apical plasma membrane. Taken together, our observations suggested that a monolayer culture of HepG2 cells can provide a useful model system to study the effects of hepatic polarity on ABCC2 localization.

Identification of associated proteins for the ABCC2 C-terminal domain

Accumulating evidence suggests that the C-terminal regions of transporter and receptor proteins anchored

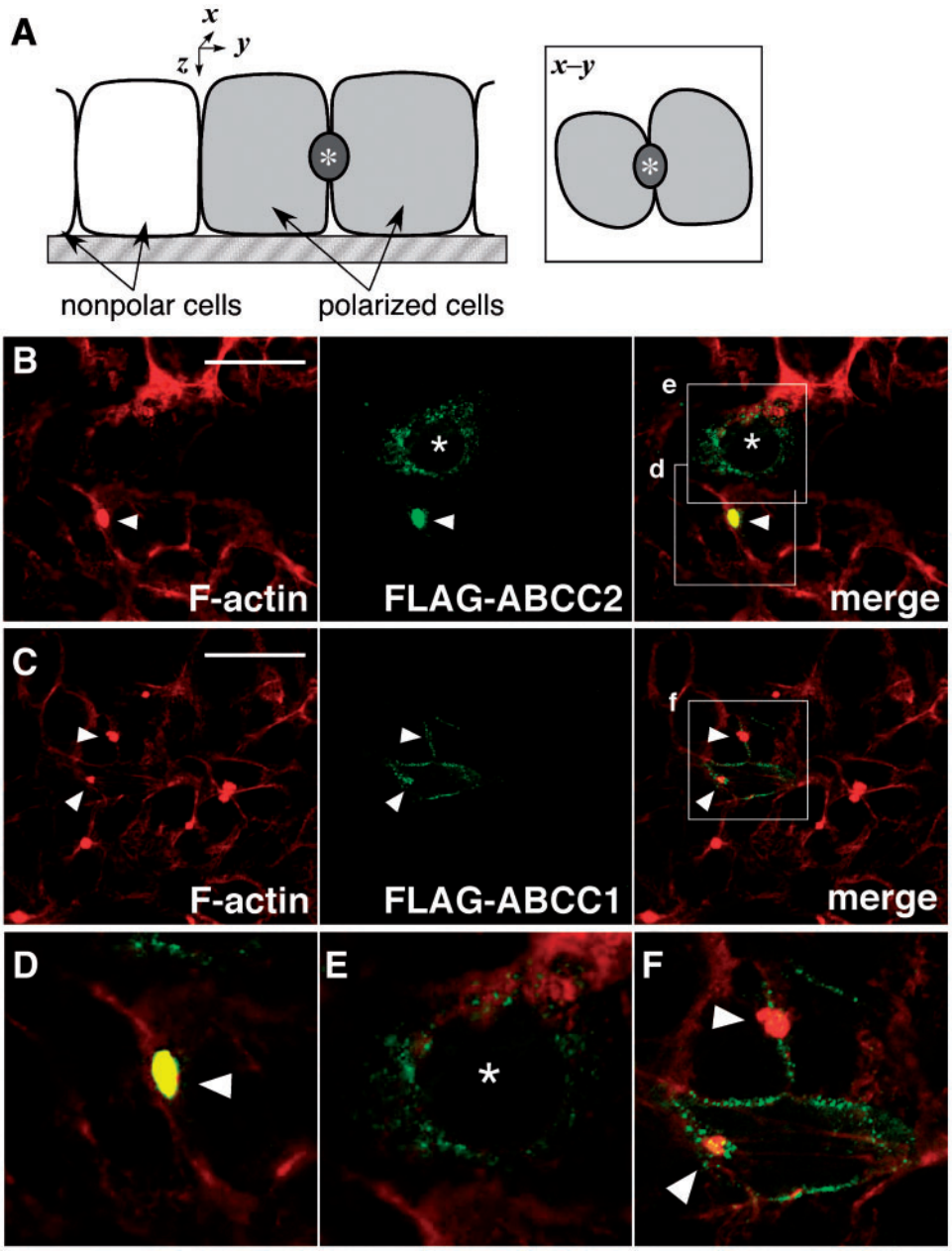

Fig. 1 Apical localization of FLAG-ABCC2 in polarized HepG2 cells. HepG2 cells develop polarity and form sealed vacuoles among the plasma membrane of two adjacent cells (A). Plasmids for FLAG-tagged proteins (0.5 μg) together with an empty vector pCMV-HA (0.5 μg) were transfected into cells. Canalicular vacuoles were visualized with rhodamine-conjugated phalloidin, which delineates a subplasmalemmal mesh-like structure of filamentous actin (F-actin). Shown are the representative immunofluorescence images of the HepG2 cells transiently expressing FLAG-tagged ABCC2 (B) and ABCC1 (C). Boxed areas (d–f) are magnified (D, E and F). The positions of apical vacuoles formed between juxtaposed polarized cells are indicated with white arrowheads. Non-polarized cells expressing FLAG-tagged proteins are indicated with asterisks. Scale bars: 20 μm.

into plasma membrane interact with adapter proteins (*12–14*). As shown in Fig. 2A, amino acid sequences of the C-terminal regions of ABCC2 are highly conserved among listed vertebrate species. This conservation implies that this region might be involved in the interaction with binding proteins necessary for the apical localization of ABCC2. To investigate this possibility, the C-terminal 77 residues of the human ABCC2 were fused with the C-terminus of GST to generate GST-CTD77, and possible binding proteins for ABCC2 were purified from HepG2 cell extracts with GST-CTD77 affinity resins (Fig. 2B). The binding specificity for the ABCC2 C-terminus was demonstrated by comparing the proteins associated with GST-CTD77 resins (lane 4), GST-alone resins (lane 3) and empty resins (lane 5). At least four polypeptide bands were resistant to high-salt wash with a buffer containing 0.5 M NaCl and therefore specific to the GST-CTD77 resin based on the stained gel. These proteins had apparent molecular weights of 70,000, 64,000, 63,000 and 51,000. Bands containing these four proteins were excised from the gel, and the gel

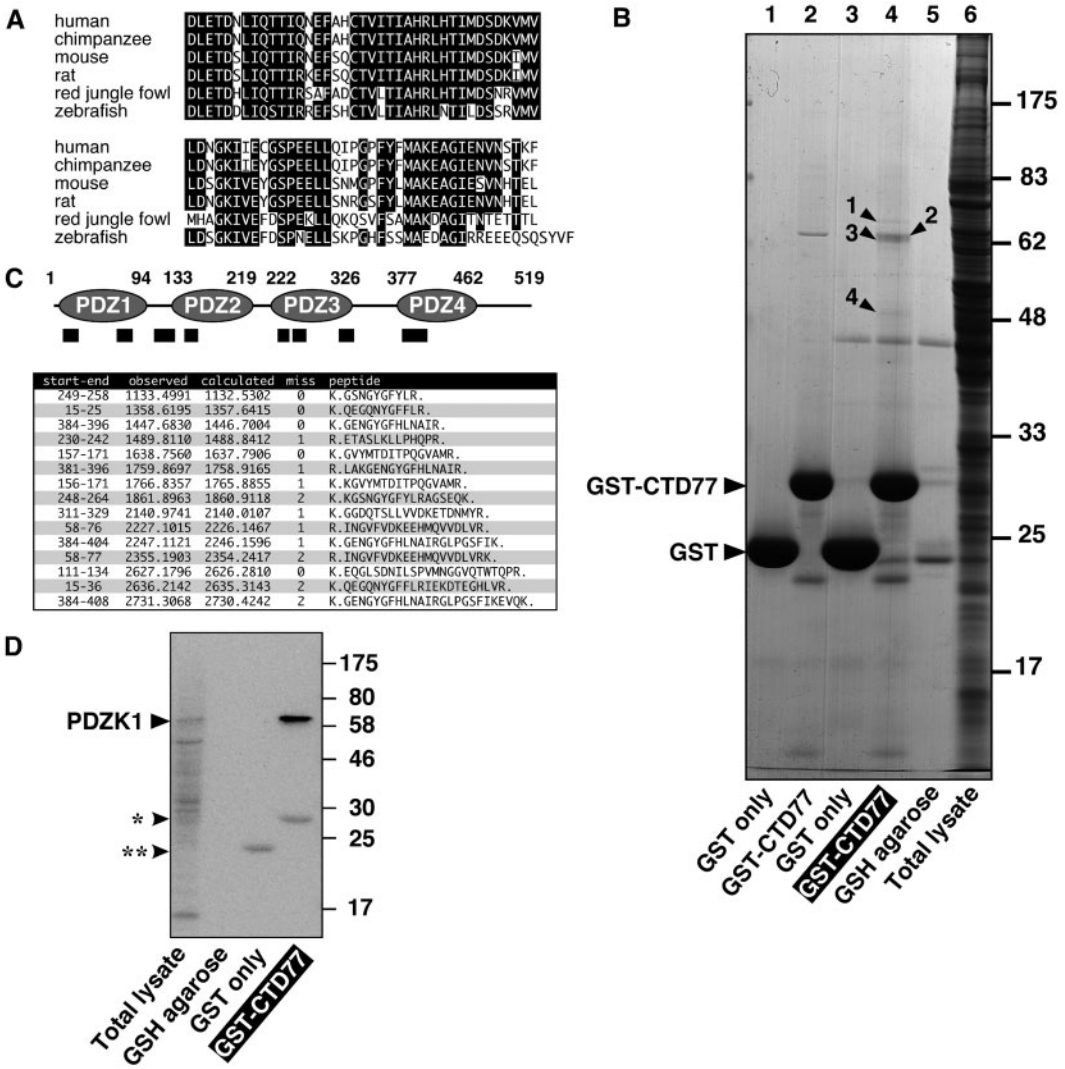

Fig. 2 Identification of binding proteins to the C-terminal tail of ABCC2. (A) Amino acid sequence alignment of the C-termini of ABCC2. The last 78 amino acids of human ABCC2 (NM000392) was compared with the homologous regions from chimpanzee (XM507976), mouse (NM013806), rat (NM012833), red jungle fowl (XM421698) and zebrafish (NM200589) ABCC2. Conserved residues are highlighted. (B) Affinity pull-down of the CTD77-associated proteins. Protein samples from different preparations were separated by SDS–PAGE and visualized by Coomassie staining. The stained gel presented here is representative of four independent experiments. Various binding proteins are indicated with numbers (lane 4). Bands pointed out by arrows were excised, in-gel trypsin digested and the peptides identified using MALDI/TOF mass spectrometry and mapped using Mascot software. Purified GST (lane 1), purified GST fusion proteins (lane 2), purified GST incubated with lysate from HepG2 cells (lane 3) and GSH–agarose resins incubated with lysate from HepG2 cells (lane 5) were loaded as controls. The positions of molecular weight standards are indicated on the right side. (C) The domain structure of PDZK1 and the locations of identified peptides. Peptides identified in the trypsinized band #3 in (B) are marked by black bars. The masses of the identified peptides are shown together with their calculated masses. (D) Confirmation of PDZK1 in the affinity purified CTD77-associated proteins by immunoblot analysis. Protein samples from different preparations were analysed by immunoblotting with anti-PDZK1 monoclonal antibodies. Two faint immuno cross-reacting bands (marked with asterisks) were likely derived from excess amounts of the bait proteins in samples.

slices were incubated with trypsin. The resulting tryptic peptides were subjected to MALDI/TOF mass spectrometry followed by database analysis using the Mascot search programme (*22*). The most prominent band of 63,000 (Fig. 2B, #3 on lane 4) showed a high correspondence with human PDZK1. Out of 58 tryptic peptides, 15 peptides proved to represent 26% of the protein sequence of PDZK1 (Fig. 2C). In addition, immunoblot analysis using PDZK1-specific monoclonal antibodies also confirmed that this 63,000 protein was PDZK1 (Fig. 2D). The other three protein bands with relative molecular masses of 70,000, 64,000 and 51,000 were identified as human Hsp70, *E. coli* Hsp70 and human β-tubulin, respectively. *Escherichia coli* Hsp70 is a minor contaminant in the purified GST-CTD77 preparation (Fig. 2B, lane 2).

Apical localization of PDZK1 in HepG2 cells

PDZK1 is predominantly expressed in kidney, liver and intestine. It is known to be primarily dispersed in cells and also shows overlapping distribution with the plasma membrane. First, we determined whether endogenous and recombinant PDZK1 proteins were expressed in HepG2 cells (Fig. 3A). Immunoblot analysis of cell extracts using monoclonal anti-PDZK1 antibody clearly demonstrated that endogenous

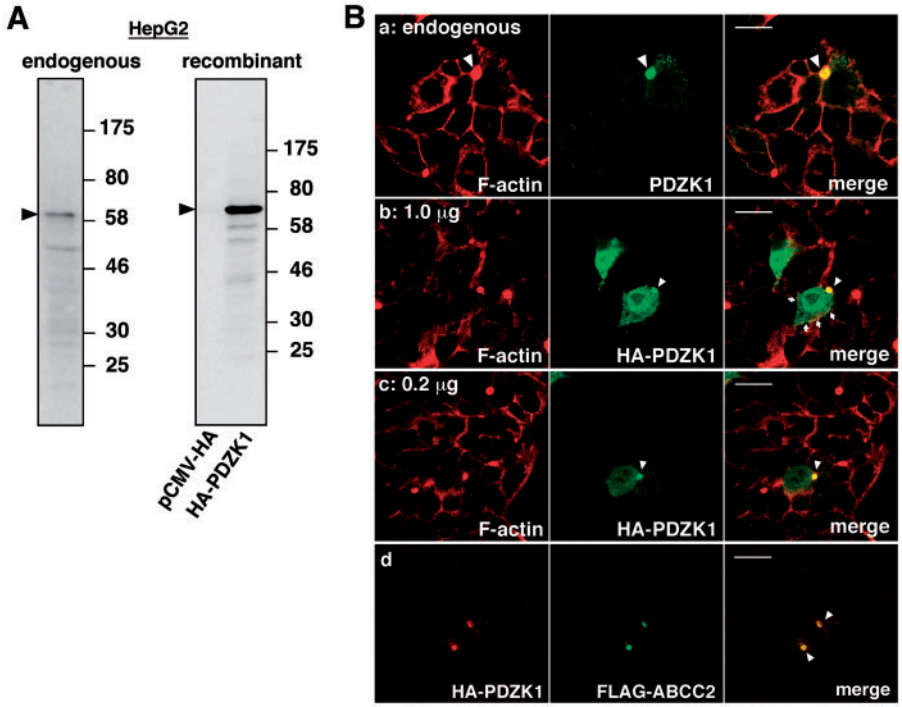

Fig. 3 Apical localization of PDZK1 in the polarized HepG2 cells. (A) Expression of PDZK1 in HepG2 cells. pCMV-HA and pHA-PDZK1 were transiently transfected into HepG2 cells and the resultant cell extracts were analysed by immunoblotting. Endogenous PDZK1 and recombinant HA-tagged PDZK1 were probed with anti-PDZK1 monoclonal antibodies and anti-HA monoclonal antibodies, respectively. The blot presented here is representative of three independent experiments. The positions of standard molecular weight markers are indicated on the right side. (B) Subcellular localization of PDZK1 in HepG2 cells. Endogenous PDZK1 was detected in cells transfected with 1.0 μg of pCMV-HA (a). Localization of HA-tagged PDZK1 were evaluated in cells transfected with 1.0 μg of pHA-PDZK1 (b) and cells transfected with 0.2 μg of pHA-PDZK1 along with 0.8 μg of pCMV-HA (c). Co-localization of HA-PDZK1 (red) and FLAG-ABCC2 (green) was also confirmed in HepG2 cells transfected with 0.5 μg of pHA-PDZK1 along with 0.5 μg of pFLAG-ABCC2 (d). Canalicular vacuoli were visualized with rhodamine-conjugated phalloidin (F-actin). Representative immunofluorescence images of the HepG2 cells transiently expressing HA-tagged human PDZK1 proteins are shown. The positions of bile canaliculi formed between two juxtaposed cells are indicated with white arrowheads. Scale bars: 20 μm.

PDZK1 was present at a detectable level in cells and had an apparent relative molecular weight of 62,000. An HA-tag was fused to the N-terminus of human PDZK1 and the resultant protein (HA-PDZK1) was expressed in HepG2 cells. Immunoblot analysis with monoclonal anti-HA antibody revealed that HA-PDZK1 gave a single major band with an apparent relative molecular weight of 66,000 and showed much higher expression levels than endogenous PDZK1. Next, we examined the subcellular distribution of PDZK1 by immunofluorescence microscopy (Fig. 3B). The signal from endogenous PDZK1 was faint but distinct throughout the cytoplasm in every cell, and the intensity of fluorescent signal varied among cells. Notably, polarized cells had punctuated staining of endogenous PDZK1 at the apical vacuoles (panel a). When 1.0 μg of the expression plasmid for HA-PDZK1 was used in transient transfection experiments, HA-PDZK1 exhibited a dense staining throughout the cytoplasm of transfected cells but still merged with the apical vacuole (panel b). Transfection with a reduced amount of expression plasmid (0.2 μg, panel c) resulted in punctuated staining of HA-PDZK1 in the apical vacuoles of transfected cells that was similar to the localization of the endogenous PDZK1. These results demonstrated that exogenously introduced PDZK1 was distributed to the apical region of HepG2 cells, like endogenously expressed PDZK1, irrespective of the amount of the transfected plasmid. Co-staining with FLAG-ABCC2 using monoclonal anti-FLAG antibody clearly demonstrated that a large proportion of the HA-PDZK1 staining overlapped with the fluorescence of FLAG-ABCC2 (panel d). Co-localization of PDZK1 and ABCC2 in the apical region of HepG2 cells was consistent with the hypothesis that an interaction between ABCC2 and PDZK1 might be involved in the apical localization of ABCC2.

Interaction of the ABCC2 C-terminus with the fourth PDZ domain of PDZK1

PDZK1 has been shown to interact with target proteins through binding either to their C-termini or, less commonly, to internal regions and to have diverse peptide binding specificity (*10*–*12*). Four classes of consensus binding motifs for the PDZ domain-containing proteins have been described (*10*, *26*), and the C-terminus of human ABCC2 (STKF-COOH) is in a class I site, which is defined by the sequence X(S/T)XΦ, where X is any amino acid and Φ is a hydrophobic amino acid. To confirm the binding site of PDZK1 in the C-terminus of ABCC2, we carried out a pull-down assay of HA-PDZK1 using purified fusion proteins. Successive substitutions of every five

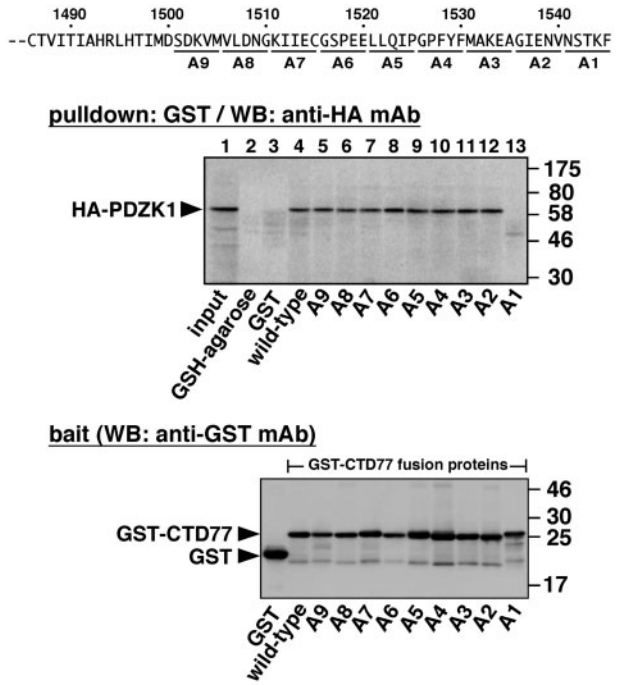

Fig. 4 Interaction between ABCC2 C-terminal tail and PDZK1. Immobilized GST fusion proteins with the C-terminal 77 amino acids tails of ABCC2 (GST-CTD77) were incubated together with lysates from HepG2 cells transiently expressing HA-tagged PDZK1. Progressive substitution of 5 amino acids for alanines are summarized on the top. Bound products were separated by SDS–PAGE followed by western blotting using anti-HA mAb. Lysate input lane accounts for 10% of the protein amount used in the pull-down reactions.

residues to an alanine pentamer were introduced to the C-terminus of GST-CTD77 to generate nine mutated CTD77 variants. These mutants were expressed in the *E. coli* BL21 strain, purified and bound on the glutathione-agarose matrices (Fig. 4, bottom panel). The resultant matrices were mixed with extracts of HepG2 cells expressing HA-PDZK1 and protein–protein interaction driven by the substituted sequences was analysed (Fig. 4). HA-PDZK1 was detected in the eluate from the wild-type GST-CTD77 matrix (lane 4), but not in the eluate from the controls, GST alone (lane 3) and empty (lane 2) matrices. A series of eight sequentially substituted constructs, starting from 1,536–1,540 (A2) to 1,501–1,505 (A9) in the C-terminus of ABCC2, resulted in roughly equivalent binding to the wild-type GST-CTD77 (lanes 5–12). In contrast, a substitution of the extreme C-terminal five residues (*1*,541–1,545) to alanine pentamer abrogated the interaction with HA-PDZK1 (A1, lane 13).

PDZK1 has four PDZ domains, which are a family of protein binding modules (*10*, *26*) that are ~90-amino acids long. To determine which region of PDZK1 is responsible for the interaction with ABCC2, the four PDZ domains were separately expressed as HA-tagged fusions in HepG2 cells (Fig. 5A). Each HA-tagged protein consists of 100–110 amino acids with an estimated molecular weight of approximately 12,000. However, HA-PDZ3 resulted in a higher molecular weight band by approximately 7,000, which arose from additional residues flanking the PDZ3. Removal of this flanking sequence markedly decreased the expression of tagged proteins, but the underlying reason for reduced expression of the shorter HA-PDZ3 remains unknown. Immunofluorescence analysis demonstrated dispersed cytoplasmic distribution of the four HA-tagged PDZ proteins in transfected cells (Fig. 5B). The dispersed cytoplasmic staining did not overlap with the apical vacuoles in contrast to the apical localization of the full-length PDZK1 in HepG2 cells (observed in Fig. 3B). Each HA-tagged PDZ domain were tested for association with the ABCC2 C-terminus by an affinity pull-down assay (Fig. 5C). Only HA-PDZ4 could be precipitated by the GST-CTD77 resin, and the binding specificity of PDZ4 to the ABCC2 C-terminus was demonstrated by its association with the GST-CTD77 resin, but not the GST-alone resin. In contrast, HA-PDZ1, HA-PDZ2 and HA-PDZ3 were not precipitated by the GST-CTD77 resin in these assays. Taken together, these results demonstrated the predominant binding of the fourth PDZ domain to the C-terminus of ABCC2. The remaining unoccupied PDZ domains of PDZK1 were available to other cellular components such as other PDZ domain-containing proteins and cytoskeletal adapter proteins.

Involvement of the PDZ4 of PDZK1 in the apical expression of ABCC2

Our findings raised a possibility that PDZK1 might retain ABCC2 in the apical plasma membrane through the direct binding of the PDZ4 domain to the C-terminus of ABCC2. To explore this possibility, excess amounts of expression plasmid encoding PDZK1 or one of the four PDZ domains (1.15 μg) were transfected into HepG2 cells with a constant amount of FLAG-ABCC2 plasmids (0.35 μg), so that the PDZK1-derived plasmids were in ~10-fold molar excess over the FLAG-ABCC2 plasmids. These transient transfections had no effect on the polarity of HepG2 cells as judged from formation of the apical vacuoles (Fig. 6A). In control cells, co-transfection with pCMV-HA did not show an inhibitory effect on the apical localization of FLAG-ABCC2 (panels a–c). In contrast, cells which were subjected to co-expression of HA-PDZ4 showed an abnormal distribution of FLAG-ABCC2 (panel g) and reduced apical localization of FLAG-ABCC2 (panels e and f, compared to panel d). For each polarized cell expressing FLAG-ABCC2, the intracellular distribution of FLAG-ABCC2 was varied, probably with respect to the amounts of co-expressed HA-PDZ4 in the transfected cells. The degree of co-localization of FLAG-ABCC2 integrated into the apical vacuoles was categorized into three groups as illustrated with yellow representing co-localization (Fig. 6B). 'Bile canalicular' localization was defined as fluorescent signal from FLAG-ABCC2 giving a single large punctuated staining that completely merged with that from the apical vacuoles. When FLAG-ABCC2 was virtually absent from the apical vacuoles in polarized cells, the distribution was represented as 'cytoplasmic', irrespective of their vesicular or reticular appearance. 'Intermediate' localization refers to partial co-localization, where FLAG-ABCC2 showed apical localization

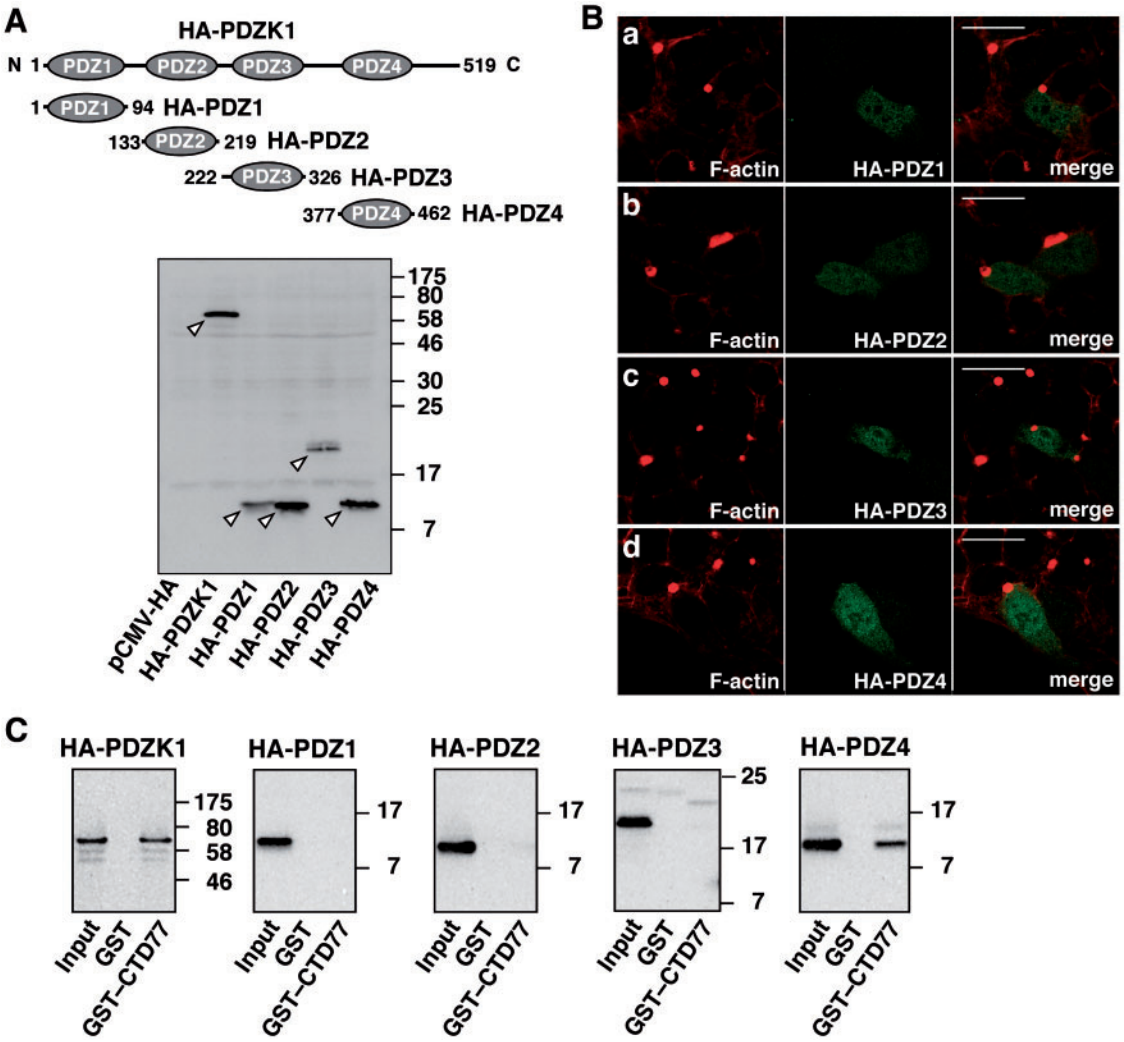

Fig. 5 Specific binding of the fourth PDZ domain of PDZK1 to the ABCC2 C-terminal tail. (A) Expression of full-length and truncated PDZK1 proteins in HepG2 cells. Schematic diagrams of the constructs for four HA-PDZ fusions are shown on the top. The amino acid positions corresponding to PDZK1-coding sequence are indicated. Cell extracts were analysed by immunoblotting with anti-HA monoclonal antibodies. The positions of standard molecular weight markers are indicated on the right side. (B) Subcellular localization of full-length HA-PDZK1 and truncated HA-PDZ domains in HepG2 cells. Representative immunofluorescence images of the HepG2 cells transiently expressing HA-tagged recombinant human PDZK1 proteins are shown. Scale bars: 20 μm. (C) The affinity pull-down assay. HA-tagged PDZK1 full-length proteins and truncated PDZ domains were transiently expressed in HepG2 cells. Lysates prepared from these cells were incubated with glutathione–Sepharose beads immobilized either with GST-CTD77 or GST. Bound products were analysed by SDS–PAGE with subsequent Western blotting using anti-HA mAb. The blot presented here is representative of three independent experiments. Lysate input lane accounts for 20% of the protein amount used in the pull-down reactions.

along with additional intracellular distribution. This 'intermediate' distribution delineates somewhat fuzzy localization with several defects in the integration into the apical vacuoles. Notably, expression of excess amounts of HA-PDZ4 over ABCC2 significantly reduced the complete 'bile canalicular' localization of ABCC2 with a reciprocal increase of intracellular accumulation of mislocalized ABCC2 ('intermediate' and 'cytoplasmic'), leading to a significant number of cells exhibiting the 'intermediate' distribution. In contrast, expression of any of the other three PDZ domains (PDZ1, PDZ2 or PDZ3), as well as full-length PDZK1, did not show any inhibitory effect on the localization of ABCC2, even when expressed at very high levels. We concluded that HA-PDZ4 inhibited the polarized distribution of ABCC2 in a competitive manner, probably based on its abundance in the transfected cells and that PDZK1 might retain ABCC2 in the apical plasma membrane through the direct binding of its fourth PDZ domain to the C-terminus of ABCC2.

To investigate the possibility that the C-terminal sequence was critical to the apical localization of ABCC2 through interaction with PDZK1, we created a series of C-terminally deleted FLAG-ABCC2 constructs and assessed the localization of the deleted ABCC2 constructs in polarized HepG2 cells (Fig. 7A). To avoid unfavourable effects that can arise from overexpression, 0.5 μg of expression plasmids were used in transient transfection experiments, and the percentage of

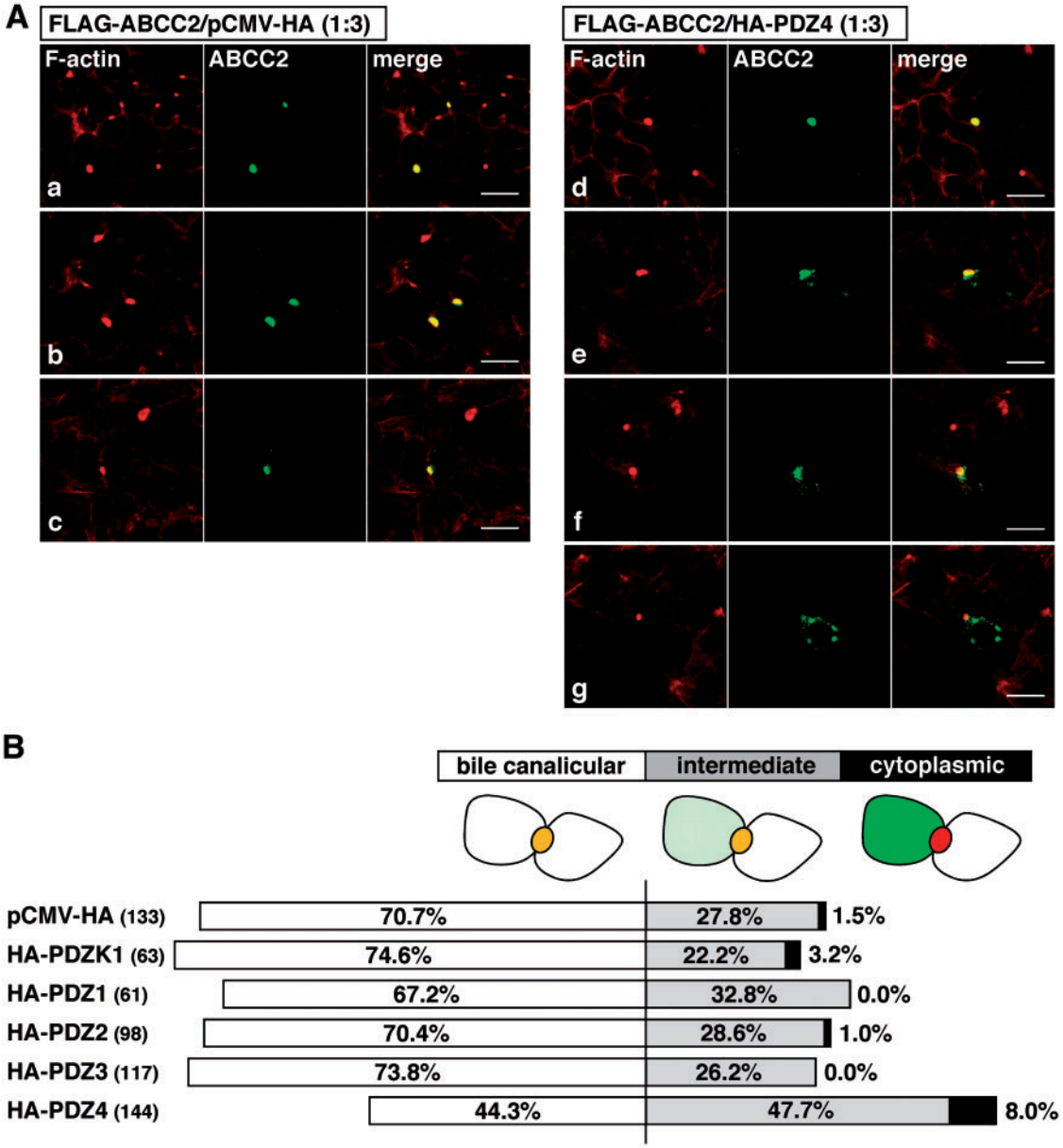

Fig. 6 Disruption of the apical localization of ABCC2 by overexpression of the fourth PDZ domain of PDZK1. (A) Effect of overexpression of the four PDZ domains of PDZK1 on the apical localization of FLAG-ABCC2 in HepG2 cells. Plasmids for FLAG-ABCC2 (0.35 μg) together with plasmids for one of the four PDZ domains (1.15 μg) were transiently transfected into cells. Representative immunofluorescence images of the HepG2 cells transiently expressing FLAG-ABCC2 are shown. Expression of excess amounts of PDZ4 reduced the correct localization of ABCC2 into the canalicular membrane (right panels, d–g) with reciprocal increase of intracellular accumulation of mislocalized ABCC2. Control cells did not show inhibitory effect on the localization of ABCC2 (left panels, a–c). Scale bars: 20 μm. (B) For each polarized cell without intense overexpression, the degree of co-localization of FLAG-ABCC2 (green) with the bile canaliculi (rhodamine-conjugated phalloidin, red) was categorized into one of three groups as illustrated in the cartoons. Yellow represents co-localization that is most similar to the normal condition. The percentage of cells displaying each localization pattern was plotted. Polarized cells were counted according to a criteria for formation of the apical vacuole (visualized by rhodamine-conjugated phalloidin), and the numbers of counted cells were given in parentheses.

cells displaying each localization pattern ('bile canalicular', 'intermediate' or 'cytoplasmic') was carefully determined after excluding all cells that exhibited dense staining of FLAG-ABCC2 throughout their cytoplasm from the analysis (Fig. 7B). Among polarized cells expressing full-length FLAG-ABCC2, 72.6% of cells exhibited complete 'bile canalicular' localization of this protein, and >97% of cells had some FLAG-ABCC2 localized to the apical vacuoles irrespective of complete or partial co-localization. In contrast, removal of the class I binding motif for PDZK1 significantly reduced the complete 'bile canalicular' localization of FLAG-ABCC2 with reciprocal increase of intracellular accumulation of mislocalized FLAG-ABCC2 (ΔCT : 1–5 and 1–10). The majority of the mislocalized proteins seemed to localize to cytoplasmic vesicles rather than have an ER-associated reticular distribution. Remarkably, this reduced

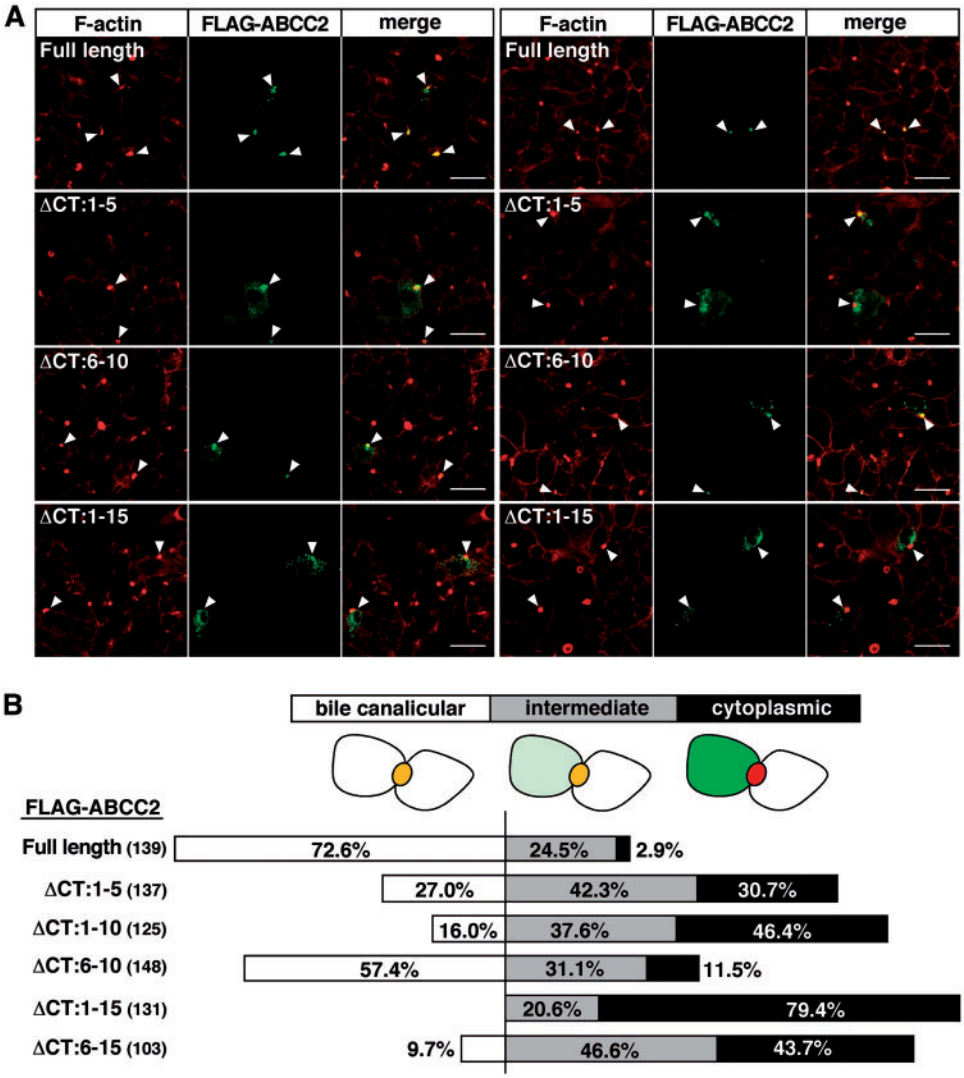

Fig. 7 Disruption of the apical localization of ABCC2 by elimination of the C-terminal PDZ-binding motif. (A) Subcellular localization of C-terminally truncated FLAG-ABCC2 in polarized HepG2 cells. Plasmids for FLAG-ABCC2 (0.5 μg) together with an empty vector pCMV-HA (0.5 μg) were transiently transfected into cells. Representative immunofluorescence images of the HepG2 cells transiently expressing FLAG-tagged proteins are shown. The positions of bile canaliculi formed between two juxtaposed cells are indicated with white arrowheads. Expression of the truncated constructs reduced the correct localization of FLAG-ABCC2 into the canalicular membrane with reciprocal increase of intracellular accumulation of mislocalized proteins. Scale bars: 20 μm. (B) For each polarized cell without excess overexpression, the degree of co-localization of FLAG-ABCC2 (green) integrated into bile canaliculi (rhodamine-conjugated phalloidin, red) was categorized into one of three groups as illustrated in the cartoons. Yellow represents co-localization that is most similar to the normal condition. The percentage of cells displaying each localization pattern was plotted. Polarized cells were counted according to a criteria for formation of the apical vacuole (visualized by rhodamine-conjugated phalloidin), and the numbers of counted cells were given in parentheses.

'bile canalicular' distribution was effectively rescued by addition of the C-terminal five residues (ΔCT : 6–10). FLAG-ABCC2/ΔCT : 6–10 was as efficiently localized to the apical vacuoles of HepG2 cells as was full-length FLAG-ABCC2. These results revealed that the class I binding motif for PDZK1 at the C-terminus of ABCC2 was critical to the apical localization of ABCC2. On the contrary, delivery to the apical vacuoles was severely impaired when 15 residues were removed from the C-terminus of FLAG-ABCC2 (ΔCT : 1–15). Among polarized cells expressing FLAG-ABCC2/ΔCT : 1–15, ~80% of cells exhibited the 'cytoplasmic' distribution of this protein, and in the remaining 20% of cells, FLAG-ABCC2/ΔCT : 1–15 was scarcely present in the apical vacuoles exhibiting the intermediate distribution. Addition of the C-terminal five residues to FLAG-ABCC2/ΔCT : 1–15 (FLAG-ABCC2/ΔCT : 6–15) could no longer rescue the abnormal distribution when compared to the effective rescue of FLAG-ABCC2/ΔCT : 6–10. As discussed by Nies and colleagues, a truncation of the C-terminus of ABCC2 by 15 amino acids may alter the native conformation to the mis-folded protein which is retained in the ER (*7*).

Discussion

In this report, we presented data on the mechanism of ABCC2 localization to the apical membrane of polarized cells. Our findings particularly focus on the

following features of ABCC2 and its interacting partner PDZK1: (i) interaction between the C-terminus of ABCC2 and the PDZ4 domain of PDZK1; (ii) co-localization of ABCC2 and PDZK1 in the apical domain of the plasma membrane; (iii) disturbance of normal apical localization of ABCC2 resulting from overproduction of the PDZ4 domain; and (iv) mislocalized ABCC2 caused by removal of its C-terminal class I PDZ-binding motif. Taken together, ABCC2 was shown to interact with PDZK1, and this interaction appeared to be important for the maintenance of the apical localization of ABCC2.

A complex array of protein–protein interactions governs many regulatory aspects of localization of newly synthesized proteins bound for the plasma membranes (*8*). In addition, the plasma membranes are subjected to rapid endocytosis and recycling, where the endocytosed proteins are sorted to lysosomes for degradation or endosomes for recycling. In spite of these dynamic movements around the plasma membranes, a significant fraction of transporter proteins, including ABCC2, are stably situated on the plasma membranes at constant levels (*27*). Adapter proteins have recently been proposed to link these transporters with other cellular components such as cytoskeletal components and kinases. PDZK1 and NHERFs are multivalent PDZ domain-containing adapter proteins. These multivalent PDZ proteins tether a variety of proteins that possess one or more PDZ-binding motifs, and also interact with each other forming networks that provide scaffolds adjacent to the plasma membrane. Transporters are hypothesized to settle within this scaffold and assemble into a macromolecular complex in the plasma membrane (*10*, *26*, *28*). Consequently, PDZ-scaffold networks may serve as signals that identify the final destination for proteins sorted to the plasma membranes. Under such circumstances, deletion of the PDZK1 gene or functional invalidation of PDZK1 proteins would result in suppression of the localization of proteins to the correct domains of the plasma membranes and eventually lead to the accumulation of mis-localized proteins.

It has been reported that PDZK1-knockout mice exhibit a decrease in several receptors and transporters, such as the high-density lipoprotein receptor (*29*) and the organic anion transporting protein 1A1 (*30*). It has also been recognized that phenotypic changes caused by inactivation of a given gene are frequently more limited than expected. For example, PDZK1-knockout mice have a normal apical localization of ABCC2 in kidney and liver (*16*). This suggests that the interaction between ABCC2 and PDZK1 is not required for the apical localization of ABCC2 or that a functional compensatory mechanism involving other adapter proteins maintains the apical localization of ABCC2 in PDZK1-deficient mice. Our study presents the following evidence that is in disagreement with the former possibility. We carried out transient transfection experiments instead of gene knock-out experiments and demonstrated that accumulation of overexpressed PDZ4 domains in HepG2 cells disturbed apical expression of ABCC2 with reciprocal increase of intracellular accumulation of mis-localized ABCC2. This result suggested that exogenously expressed PDZ4 domains out competed the intrinsic PDZK1 proteins as well as other adapter proteins that may have compensated for the loss of PDZK1, and consequently may have disturbed the correct localization of ABCC2 in a competitive fashion. Our findings strongly suggested that PDZK1 was involved in the apical localization of ABCC2 through the binding of its fourth domain to the C-terminus of ABCC2.

Based on previous findings and the findings of this study, we propose a model depicting the coupling of ABCC2 with adapter proteins in the regulation of the apical localization of ABCC2. Highly specialized compartments are assumed to form underneath the apical plasma membranes and contain a network of scaffolding proteins such as cytoskeletal F-actin, radixin, NHERF1 and PDZK1. Radixin is the major ERM family protein in hepatocytes and crosslinks actin filaments to various cellular components. PDZK1 and NHERF1 interact with each other and constitute the proposed scaffolding network that can tether a variety of membrane proteins with overlapping specificities. It is noteworthy that human organic anion transporter isoform 4 shows higher binding affinity for PDZK1 ($K_D = 36$ nM) than for NHERF1 ($K_D = 41.7$ μM) (*31*). The presence of a similar C-terminal PDZ-binding motif (STSL-COOH) like ABCC2 in human organic anion transporter isoform 4 predicts that PDZK1 may connect ABCC2 with higher affinity than does NHERF1. Our pull-down experiments actually demonstrated tight binding of PDZK1 to ABCC2 because their association was resistant to intensive washing with a high-salt buffer. Accordingly, it is feasible that the C-terminal PDZ-binding site of ABCC2 associates with the fourth PDZ domain of PDZK1, leaving the remaining unoccupied PDZ domains available to NHERF1 and other cellular components. NHERF1 contains an additional C-terminal ERM-binding domain and links the PDZ scaffolding networks via radixin to the cytoskeleton.

Assembly of such macromolecular complexes can provide a potential anatomical basis for why drastic changes in ABCC2 localization are not seen in PDZK1-knockout mice. Radixin plays a fundamental role in crosslinking of scaffolding networks to cytoskeletons; consequently, the hepatocanalicular localization of ABCC2 is severely disrupted in radixin knockout mice (*9*). Binding of PDZK1 is likely to be in close proximity to the C-terminus of ABCC2. However, other adapter proteins such as NHERF1 and radixin can compensate for the absence of PDZK1 only in PDZK1-deficient mice, so that PDZK1 knockout mice have a normal apical localization of ABCC2 (*16*). Similarly, a recent report described that deletion of the NHERF1 gene did not cause mislocalization of ABCC2 but did result in decreased expression of ABCC2 at the apical plasma membrane (*32*). Therefore, other adapter proteins, such as radixin and PDZK1, may compensate for the loss of NHERF in NHERF1-deficient cells. Such a macromolecular complex may provide functional redundancy in the apical scaffolding network and can explain the absence of significant phenotypic changes

in PDZK1-knockout mice. Double knockout of both PDZK1 and NHERF1 may reveal an overlap in their function and specificities.

Apical compartmentalization of the macromolecular complexes containing ABCC2 and PDZK1 may have additional physiological implications. Ser-509 of PDZK1 is phosphorylated by a cAMP-dependent protein kinase, and this phosphorylation is required for up-regulation of high-density lipoprotein receptor in hepatoma cells (*33*). Interestingly, cAMP is known to induce acute enhancement of exocytic insertion of ABCC2 into the apical membrane in rat hepatocytes (*34*, *35*). PDZK1 may, therefore, have a function in regulating cAMP-responsive turnover of ABCC2. Further investigation is necessary to assess this interesting possibility. ABCC7 is a member of the ABC transporter family C and interacts with PDZK1 and NHERF1. PDZK1 binds two ABCC7 molecules simultaneously using its third and fourth PDZ domains and, thereby, regulates dimer formation and chloride channel activity of ABCC7 (*22*, *36*). Deletion of the PDZ-binding motif at the C-terminus of ABCC7 did not affect its localization to the apical membrane, but the deletion did decrease endocytic recycling of ABCC7 accompanied by a shortened half-life of ABCC7 (*37*). Similarly, the class I PDZ-binding motif of β_2-adrenergic receptor was revealed to promote the efficient sorting of internalized receptors to recycling endosomes (*38*). These findings indicate that interaction between PDZK1 and ABCC2 might facilitates post-endocytic recycling of endocytosed ABCC2 back to the apical membrane under normal conditions. In the absence of this interaction, ABCC2 might accumulate in vesicles inside the cell, a hypothesis that is consistent with our results. Therefore, it is probable that defects in PDZK1 might decrease endocytic recycling of ABCC2 to the apical plasma membrane and consequently increase lysosomal degradation. Further studies should examine the half-life and endocytic recycling of ABCC2 in model cell lines expressing mutant ABCC2 with altered or deleted PDZ-binding motifs.

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Conflict of interest

None declared.

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