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Involvement of a di-leucine motif in targeting of ABCC1 to the basolateral plasma membrane of polarized epithelial cells

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ABSTRACT

Localization of ATP-binding cassette transporter isoform C1 (ABCC1) to the basolateral membrane of polarized cells is crucial for export of a variety of cellular metabolites; however, the mechanism regulating basolateral targeting of the transporter is poorly understood. Here we describe identification of a basolateral targeting signal in the first cytoplasmic loop domain (CLD1) of human ABCC1. Comparison of the CLD1 amino acid sequences from ABCC1 to ABCC2 revealed that ABCC1 possesses a characteristic sequence, E^{295}EVEALI^{301}, which is comprised of a cluster of acidic glutamate residues followed by a di-leucine motif. This characteristic sequence is highly conserved among vertebrate ABCC1 orthologs and is positioned at a site that is structurally equivalent to the apical targeting signal previously described in ABCC2. Alanine scanning mutagenesis of this sequence in full-length human ABCC1 showed that both L^{300} and I^{301} residues were required for basolateral targeting of ABCC1 in polarized HepG2 and MDCK cells. Conversely, E^{295}, E^{296}, and E^{298} residues were not required for basolateral localization of the transporter. Therefore, a di-leucine motif within the CLD1 is a basolateral targeting determinant of ABCC1.

1. Introduction

ATP-binding cassette (ABC) transporters comprise a large family of multi-spanning transmembrane proteins that are divided into seven subfamilies (A–G). ABC transporters are found in various membranous organelles, where they vectorially transport a variety of endogenous and exogenous compounds in an ATP-dependent manner [1]. Human ABC transporter subfamily C (ABCC) contains 13 isoforms of plasma membrane-associated cellular export pumps, including eight multidrug efflux transporters [2]. ABCC1 was originally reported as a multidrug resistance-associated protein that is expressed throughout normal and malignant tissues; the protein transports a wide variety of substances, including hydrophobic compounds and organic anion conjugates [3]. ABCC isoforms are composed of two tandemly arranged polytopic membrane spanning domains (MSD1 and MSD2) and two cytoplasmic nucleotide binding domains (NBD1 and NBD2). An additional membrane spaning domain (MSD0) at the N-terminus is connected to MSD1 by the first cytoplasmic loop domain (CLD1) (Fig. 1A).

Although ABCC1 and ABCC2 share structural similarities, the proteins are differentially localized to the basolateral and apical surfaces of polarized epithelial cells, respectively [2]. Epithelial cells form a boundary between different extracellular environments; these cells exhibit polarity by differentiating their plasma membranes into apical and basolateral domains. Maintenance of a polarized distribution of membrane proteins is crucial for epithelial cell homeostasis, as well as for vectorial transport of a wide variety of metabolites. Despite the physiological importance of proper ABCC1 localization, the molecular mechanism responsible for basolateral targeting of ABCC1 in polarized epithelial cells is poorly understood. In addition, the retention and recycling mechanisms, which determine the further intracellular trafficking of delivered proteins, are also unconfirmed. Several attempts to identify the distinct protein regions required for basolateral targeting of ABCC1, using mutagenesis or analysis of chimeras formed between ABCC1 and ABCC2, have shown that the CLD1 [4–6], as well as a cooperation between the C-terminus and MSD0 [7], are crucial for proper trafficking of ABCC1. However, the specific targeting signals that direct ABCC1 to the basolateral plasma membrane are currently unknown.

Our previous research efforts have focused on determining the subcellular localization of ABC transporters and on elucidating targeting pathways that direct proteins to particular organelles [8–12]. We recently described a five amino acid apical targeting sequence (ATS) within the CLD1 of human ABCC2 (S^{283}QDAL^{287}),

Abbreviations: ABCC1, ATP-binding cassette transporter isoform C1; APN, aminopeptidase-N; ATS, apical targeting sequence; CLD, cytoplasmic loop domain; HA, hemagglutinin; MCC, Manders' correlation coefficient; MSD, membrane spanning domain; Na,K-ATPase, Na^+,K^+-ATPase α1 subunit; NBD, nucleotide binding domain.

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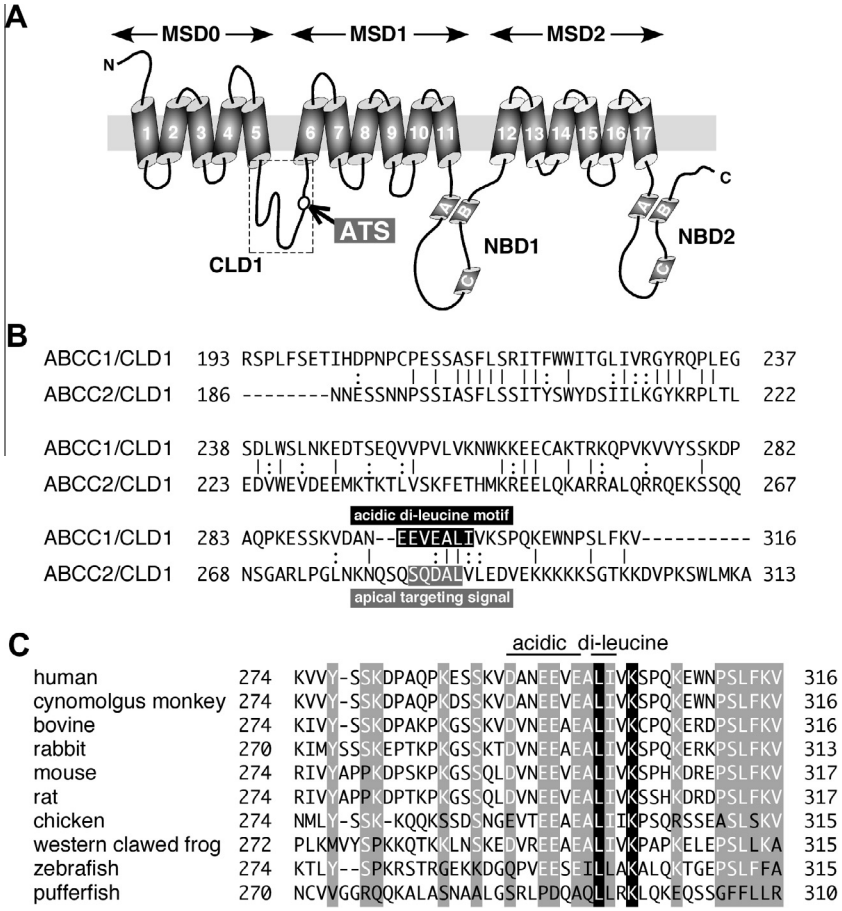

Fig. 1. A possible basolateral targeting signal in the CLD1 domain of ABCC1. (A) Predicted membrane topology of ABCC1, performed using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). ATS is the apical targeting signal of ABCC2. The CLD1 domain is indicated by a dashed box. Walker A and B motifs and the family signature C present in the NBD1 and NBD2 domains are also indicated. (B) Comparison of CLD1 amino acid sequences between ABCC1 and ABCC2. Identical amino acids and conserved residues are indicated by vertical lines and colons, respectively. The apical targeting region of ABCC2 is replaced by a cluster of acidic glutamate residues followed by a di-leucine motif in the ABCC1 sequence. (C) Amino acid sequence alignment of CLD1 from various species. The 43 amino acid CLD1 region of human ABCC1 (NM_004996) was compared with the identical regions within ABCC1 homologs from cynomolgus monkey (AY146672), bovine (AB082124), rabbit (G1TVN1), mouse (AF022908), rat (NM_022281), chicken (AJ851786), western clawed frog (NM_001127001), zebrafish (XM_002661202), and pufferfish (H2U148). Residues identical to those found in the human ABCC1 sequence are highlighted by white letters. Dark gray shading indicates residues conserved in all species; light gray shading indicates residues conserved in seven or more species.

which represents a different class of sorting signal and controls targeting of ABCC2 to the apical membrane of polarized epithelial cells [12]. Given that ABCC1 and ABCC2 display comparable membrane topologies, we hypothesized that the CLD1 of ABCC1 may contain sorting signals at a similar location to the ATS of ABCC2. In the present study, we analyzed this region in ABCC1 and found that two amino acids, L^{300} and I^{301}, form an important basolateral targeting determinant of ABCC1 in polarized cells.

2. Materials and methods

2.1. Construction of plasmids

A human ABCC1 cDNA clone was provided by Dr. S. Kato through distribution by the RIKEN BioResource Center (clone ID: RBb11B10). The entire coding sequence of ABCC1 was amplified by polymerase chain reaction using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA). The primer sequences are listed in Supplementary Table S1. The amplified DNA fragment was digested with NotI and XbaI restriction enzymes; the digested fragment was then cloned into the pRc/CMV-HA plasmid [8], to create pRc/hABCC1-HA. This plasmid expresses C-terminally hemagglutinin-tagged ABCC1 (ABCC1-HA) in transfected cells. Site-directed *in vitro* mutagenesis of ABCC1 was performed using the QuickChange kit (Stratagene) and primers listed in Supplementary Table S1. The nucleotide sequences of the mutants were confirmed by dideoxy sequencing of the respective DNA fragments using a Prism 3100 Avant sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Expression of recombinant proteins in cultured cells

HepG2 cells and MDCK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, at 37 °C under a 5% CO_2 atmosphere. Cells were transfected using FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Transfection of HepG2 cells was performed as described previously [12,13]. MDCK cells (1×10^5 cells) were seeded into a 3.5 cm dish; 8 h after plating, cells were incubated with the DNA-lipid complex for 24 h, washed once with prewarmed culture medium, and then incubated in fresh medium for an additional 40 h.

2.3. Analysis of the subcellular localization of ABCC1-HA in polarized cells

Immunofluorescence studies were performed as described previously [9]. To detect the epitope-tagged proteins by immuno-

staining, transfected cells were incubated for 60 min with either a 1:4000 dilution of anti-HA monoclonal antibody (Covance, Princeton, NJ) or a 1:2000 dilution of anti-FLAG monoclonal antibody (Sigma, St. Louis, MO, USA), followed by a 1:1000 dilution of Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) for a further 60 min. To analyze the colocalization of ABCC1-HA with apical and basolateral membrane markers, monoclonal antibodies targeting aminopeptidase-N (APN) (R&D Systems, Minneapolis, MN, USA) and Na^+,K^+-ATPase α1 subunit (Na,K-ATPase) (Abcam, Cambridge, UK) were used, respectively. To detect the tagged proteins in this colocalization analysis, anti-HA polyclonal antibody (1:5000 dilution; Covance) and Alexa Fluor 488-conjugated anti-rabbit IgG (1:2000 dilution; Invitrogen) were used. Immunofluorescence was observed using a LSM5-PASCAL confocal laser scanning microscope (Carl Zeiss, Jena, Germany) as described previously [11,12].

To assess polarity, HepG2 cells were stained with rhodamine-conjugated phalloidin (Invitrogen); it has previously been reported that apical vacuoles formed between adjacent cells can be visualized using rhodamine-conjugated phalloidin [14]. For each transfection, cells that displayed both polarization and ABCC1-HA expression, as indicated by punctuate rhodamine fluorescence and Alexa Fluor 488 fluorescence, respectively, were examined. To limit the undesirable effects of protein overexpression, reduced amounts of expression plasmids (typically 0.5 μg) were used for transient transfection, and polarized cells exhibiting dense staining of ABCC1-HA throughout the cytoplasm were excluded from the localization analysis. Three independent transfections and observations were performed.

The extent of colocalization of ABCC1-HA with apical and basolateral membrane marker proteins was measured quantitatively by Manders' correlation coefficients (MCCs) using Fiji software with a Coloc_2 plug-in (http://fiji.sc/wiki/index.php/Colocalization_Analysis) as described previously [12]. Regions of interest were selected using the freehand selection tool. According to a practical guide [15], MCC is reportedly a more suitable measure of colocalization than Pearson's colocalization coefficient when the fluorescent signals distribute to different types of compartments. Two different MCC values (M_1 and M_2) describe the independent contributions of two selected channels to the pixels of interest; M_1 represents the fraction of red signal in compartments containing green signal and M_2 accounts for the fraction of green signal in compartments containing red signal. MCC values range from zero (uncorrelated distributions of two probes with one another) to one (perfect colocalization of two images).

3. Results

3.1. A basolateral targeting signal resides in the first cytoplasmic loop domain of ABCC1

ABCC1 contains three long cytoplasmic loop domains (CLD1, NBD1, and NBD2) and six short cytoplasmic loops (Fig. 1A). NBD1 and NBD2 are approximately 300 amino acids in length and are required for ATP-binding. The CLD1 domain is a comparatively short polypeptide of 124 amino acids (amino acid residues 193–316, where residue 1 is the initiation methionine). ABCC1 and ABCC2 are closely related members of the ABCC family that display a similar membrane topology [2]. We previously demonstrated that an ATS (S^{283}QDAL^{287}) within the CLD1 of ABCC2 is required for apical targeting of ABCC2 in polarized cells [12]; therefore, we postulated that the CLD1 of ABCC1 may be involved in its basolateral targeting.

To explore this possibility, the CLD1 amino acid sequences from ABCC1 and ABCC2 were compared (Fig. 1B). Sequence similarity was higher in the N-terminal half than the C-terminal half of CLD1. Notably, the C-terminal half was found to harbor a characteristic sequence that includes a cluster of acidic glutamate residues followed by a di-leucine motif; this sequence is situated in the position equivalent to that of the ATS in ABCC2. In addition, sequence alignments of the C-terminal half of the ABCC1 CLD1 domain from a number of vertebrate species demonstrated broad conservation of this characteristic sequence, with a consensus of [D/E]XXXL[L/I]. This result suggests involvement of the characteristic sequence in the physiologically important basolateral targeting of ABCC1 (Fig. 1C). To date, a number of basolateral targeting signals have been identified, many of which resemble endocytic motifs [16–18]. For example, tyrosine-based motifs (NPXY or YXXΦ, where X represents any amino acid and Φ denotes a large hydrophobic residue) and bipartite sorting signals of hydrophobic residues (such as di-leucine motifs, represented as L[L/I]) have been shown to function as trafficking signals for many basolateral membrane proteins. We therefore sought to determine the exact nature of the basolateral targeting signal in the CLD1 of ABCC1; accordingly, we focused on the newly identified conserved sequence and its neighboring residues.

3.2. L^{300} and I^{301} are key residues for basolateral targeting of ABCC1 in polarized HepG2 cells

Like hepatocytes, HepG2 cells are structurally and functionally polarized. The apical vacuoles and cell perimeters are readily visualized in polarized cells under a fluorescence microscope using rhodamine-conjugated phalloidin as described previously [11,12]. Immunostaining of ABCC1-HA was localized exclusively to the cell perimeters of HepG2 cells, excluding the apical vacuoles (Fig 2A). The staining pattern of ABCC1-HA overlapped with the staining pattern of the basolateral membrane marker Na,K-ATPase (Fig. 3A), confirming rigid basolateral localization of ABCC1-HA. FLAG-tagged ABCC2 produced a single punctated signal between neighboring HepG2 cells that merged with the apical vacuoles and APN signal (Fig. 2B), indicating apical localization of the protein.

To determine the relative importance of individual residues within the conserved ABCC1 CLD1 sequence, we generated ABCC1-HA mutants harboring alanine substitutions at nine amino acid positions within the conserved sequence and neighboring residues (E^{295}EVEALIVK^{303}). Two ABCC1-HA point mutants, L300A and I301A, displayed a mixed distribution pattern, indicating diminished basolateral targeting specificity. In addition to their usual localization to the basolateral membrane, significant proportions of the L300A and I301A mutants were found in the apical vacuoles of every polarized HepG2 cell. All other ABCC1 point mutants tested exhibited normal basolateral localization, which excludes the possibility that perturbed localization of the L300A and I301A mutants was caused by gross protein misfolding arising from a point mutation and inhibition of ABCC1 trafficking to the basolateral membrane. To determine the role of the three acidic glutamate residues in the nine-amino acid sequence E^{295}EVEALIVK^{303}, additional mutants containing various combinations of alanine substitutions of these three residues were generated (Supplementary Fig. S1C). The EA-1, EA-2, and EA-3 mutants were created by substitution of different combinations of two of the three glutamate residues; the EA-4 mutant was generated by substitution of all three glutamate residues. Confocal micrographs demonstrated that none of these mutations affected basolateral trafficking of ABCC1-HA in HepG2 cells (Supplementary Fig. S1A).

To evaluate the extent of colocalization of the wild-type and mutated forms of ABCC1-HA with apical and basolateral membrane markers, MCCs were calculated. The M_1 and M_2 values between APN and ABCC1-HA were 0.08 ± 0.03 and 0.06 ± 0.02, respectively. Conversely, the M_1 and M_2 values between Na,K-ATPase and ABCC1-HA were 0.64 ± 0.05 and 0.69 ± 0.04, respectively

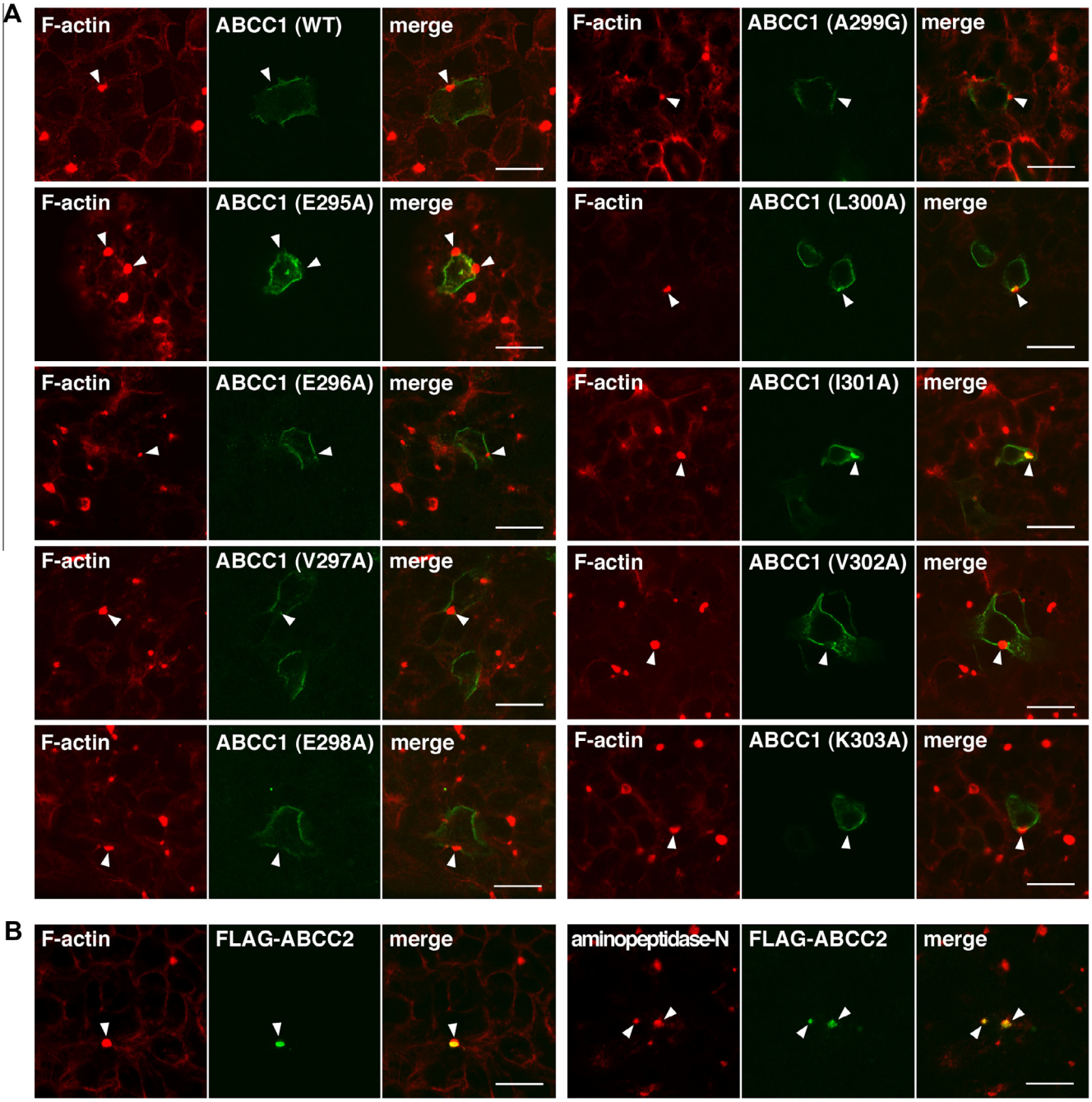

Fig. 2. Identification of an ABCC1 basolateral targeting signal. (A) A series of alanine scanning mutants were generated by replacing residues 295–303 in the CLD1 region of ABCC1. HepG2 cells were transiently cotransfected with 0.5 μg of the indicated ABCC1-HA mutant expression plasmids and 0.5 μg of empty vector pCMV-HA. HepG2 cells transiently expressing wild-type or mutant ABCC1 were fixed, permeabilized with 0.2% Triton X-100, and then immunostained with anti-HA mAb. Fluorescence colocalization images were acquired for each ABCC1 variant (green) following staining of the basolateral perimeters and apical vacuoles with phalloidin (red, F-actin). Representative merged immunofluorescence images of the transfected cells are shown. The positions of apical vacuoles formed between juxtaposed polarized cells are indicated by white arrowheads. Scale bar, 20 μm. Only two variants, L300A and I301A, exhibited a mixed localization pattern. Results of intracellular localization of HA-tagged alanine-scanning mutants in polarized HepG2 cells are also summarized in Supplementary Fig. S1. (B) HepG2 cells were fixed and permeabilized with 0.2% Triton X-100. FLAG-tagged ABCC2 was stained with anti-FLAG mAb (green). As a control, the apical plasma membranes was visualized with anti-APN mAb (red). The positions of apical vacuoles formed between juxtaposed polarized cells are indicated by white arrowheads. Scale bar, 20 μm.

(Fig. 3A). These data indicate uncorrelated distribution of APN and ABCC1-HA, as well as colocalization of Na,K-ATPase and ABCC1-HA at the basolateral membrane. The MCCs of the L300A mutant against APN and Na,K-ATPase were 0.59 ± 0.06 and 0.62 ± 0.08, respectively, indicating a mixed apical and basolateral localization pattern (Fig. 3B). Similarly, MCCs of the I301A mutant were 0.63 ± 0.05 and 0.59 ± 0.04, respectively, which also suggests a mixed localization of the I301A mutant (Fig. 3C). Overall, we concluded that the di-leucine motif $L^{300}I^{301}$ is required for correct targeting of human ABCC1 to the basolateral membrane of polarized HepG2 cells.

3.3. L^{300} and I^{301} are important for basolateral localization of ABCC1 in MDCK cells

MDCK cells are columnar epithelial cells and form an apical domain at the cell apex. To determine whether the observed trafficking of ABCC1 to the basolateral membrane in polarized HepG2 cells

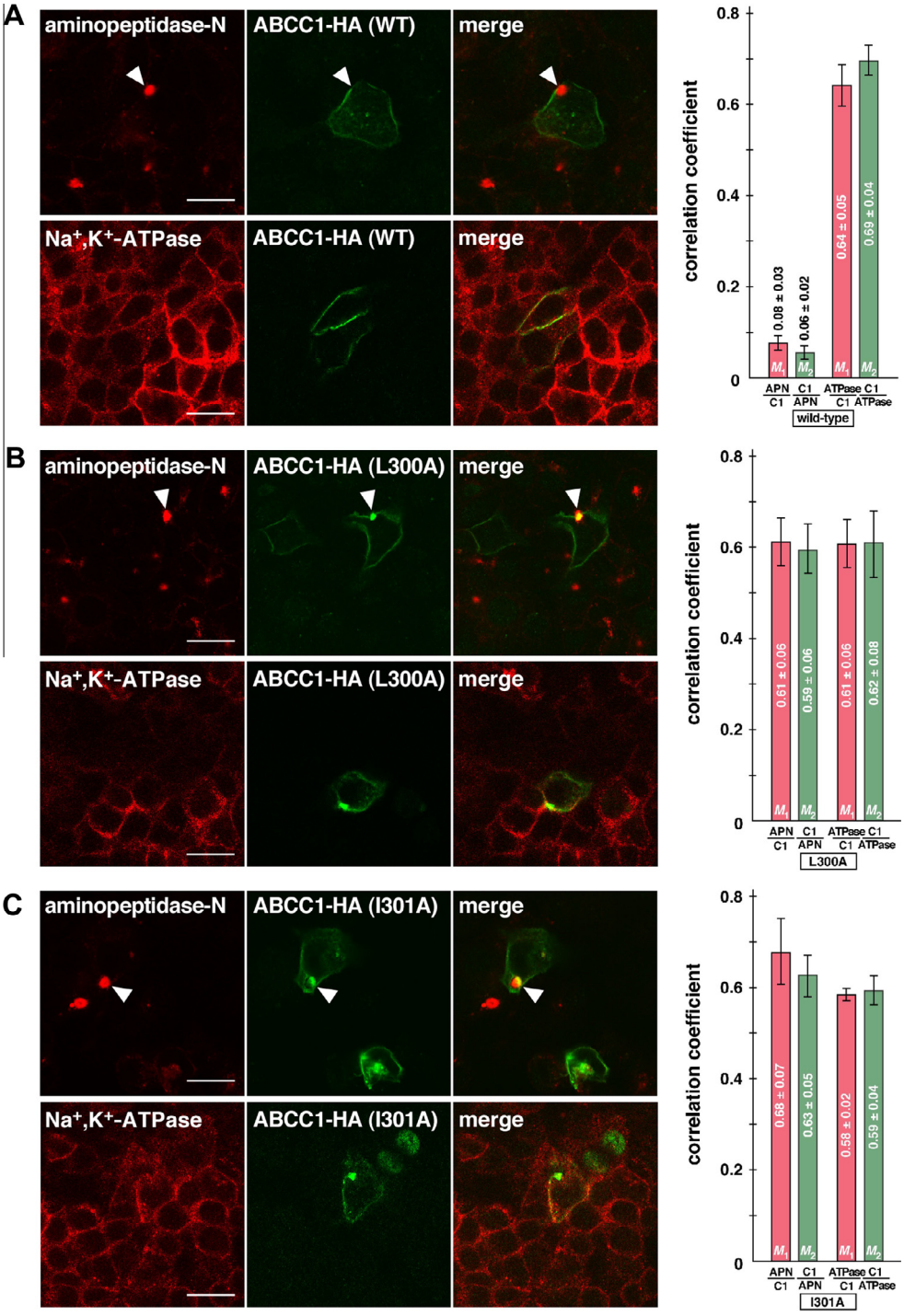

Fig. 3. Colocalization of wild-type and mutant ABCC1-HA proteins with apical and basolateral markers. HepG2 cells were transiently cotransfected with 0.5 μg of the wild-type (A), L300A (B), or I301A (C) ABCC1-HA expression plasmids, along with 0.5 μg of empty vector pCMV-HA. Cells were fixed and permeabilized with 0.2% Triton X-100. HA-tagged proteins were stained with anti-HA mAb (green). As a control, the apical vacuoles and the basolateral membranes were visualized with anti-APN mAb and anti-Na,K-ATPase mAb, respectively (red). Representative merged immunofluorescence images of the transfected cells are shown. The positions of apical vacuoles formed between juxtaposed polarized cells are indicated by white arrowheads. Scale bar, 20 μm. MCC values (M_1 and M_2) are presented as mean ± S.D.

is also observed in MDCK cells, we generated transient transfectants using the same set of ABCC1 mutants (Fig. 4 and Supplementary Fig. S1B). As expected, ABCC1-HA was readily detected at the basolateral membrane of MDCK cells. The L300A and I301A mutants lost their basolateral-specific targeting and produced a mixed staining pattern at both the apical and basolateral sides (Fig. 4). All other mutants retained basolateral-specific localization. In summary, MDCK cells displayed trafficking profiles of ABCC1-HA that were similar to those observed in polarized HepG2 cells.

4. Discussion

The polarized distribution of ABCC1 is physiologically important for the maintenance of vectorial metabolite transport [2,3]. In this study, we demonstrate that the $L^{300}I^{301}$ di-leucine motif of ABCC1 is important for basolateral targeting of the transporter. In general, removal of a basolateral targeting signal often results in exclusive apical localization of the modified protein [16–18]. However, our results showed that inactivation of the ABCC1 di-leu-

cine motif resulted in mixed sorting of the protein, which is suggestive of a nonpolarized default mechanism that has little preference for either the apical or the basolateral domain. Therefore, this di-leucine motif is a likely determinant of the basolateral targeting of ABCC1. The presence of basolateral targeting information in the C-terminal region of ABCC1, which acts in cooperation with the N-terminus, was initially predicted during analysis of hybrid proteins formed between ABCC1 and ABCC2 [7]. Other unidentified regions of ABCC1 may also contain auxiliary information required for proper basolateral localization; these regions may combine and become functional in the native conformation of ABCC1.

Many basolateral membrane proteins are sorted by signals resembling endocytic motifs, via interaction with clathrin and clathrin-associated adaptor proteins. It is widely accepted that basolateral trafficking requires capture of the proteins into clathrin-coated vesicles via interaction of clathrin adaptor AP-1B with their sorting signals [16,19,20]. However, many aspects of clathrin-mediated sorting of basolateral proteins remain unclear; in particular, polarized epithelial cells diverge with respect to how they deliver their basolateral proteins to the plasma membrane. For example, MDCK cells express AP-1B for sorting of proteins to the basolateral membrane, whereas HepG2 cells and LLC-PK1 cells do not [19,21]. In spite of this difference, our results demonstrated a rigid basolateral localization of HA-tagged ABCC1 in both HepG2 cells and MDCK cells. Interestingly, a recent report [21] demonstrated a role of the commonly expressed AP-1A clathrin adaptor complex in basolateral protein sorting at the trans-Golgi network, which is complementary to the established role of AP-1B in basolateral sorting at recycling endosomes. Therefore, the similar sorting behaviors of ABCC1 reported in HepG2 and MDCK cell lines in this study may be attributed to either a basolateral localization mechanism independent of AP-1B or to a differential interpretation of combinations of ABCC1 targeting motifs by the two cell lines. An understanding of how the $L^{300}I^{301}$ di-leucine motif is interpreted and the molecular machinery that interacts with ABCC1 remains to be determined. Defining the proteins that bind to ABCC1 should provide insights into the molecular mechanism of polarized distribution of this physiologically important transporter in epithelial cells.

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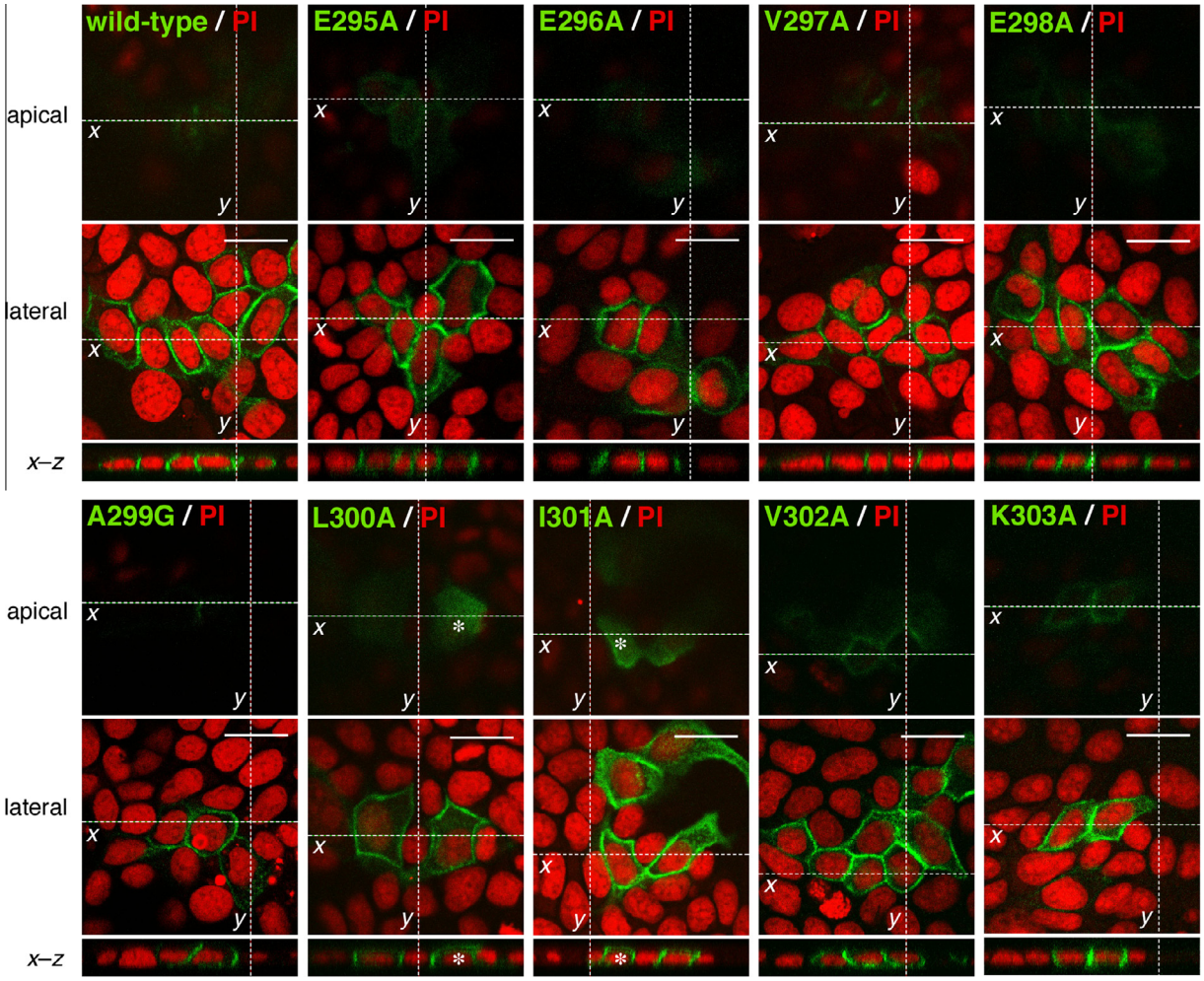

Fig. 4. Subcellular distribution of ABCC1-HA in MDCK cells. MDCK cells expressing wild-type ABCC1-HA or the indicated ABCC1-HA mutants were immunostained with anti-HA mAb (green). Nuclei were stained with propidium iodide (PI, red) and representative merged images are shown. Single merged panels of representative apical, lateral, and *x–z* sections are shown. The L300A and I301A mutants displayed a mixed localization to the apical and basolateral membranes. The positions of the apical membranes as observed in the *x–z* section are indicated by asterisks. Scale bar, 20 μm. Results of intracellular localization of HA-tagged alanine-scanning mutants in polarized MDCK cells are summarized in Supplementary Fig. S1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.013.

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