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Involvement of NHERF1 in apical membrane localization of MRP4 in polarized kidney cells

Md. Tozammel Hoque, Gwenaëlle Conseil, Susan P.C. Cole *

Division of Cancer Biology & Genetics, Cancer Research Institute, 10 Stuart St., Queen's University, Kingston, ON, Canada K7L 3N6

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ABSTRACT

Multidrug resistance protein 4 (MRP4/ABCC4), a member of the ATP-binding cassette protein superfamily, confers resistance to nucleoside and nucleotide analogs as well as camptothecin derivatives. MRP4 also mediates the efflux of certain cyclic nucleotides, eicosanoids, conjugated steroids, and uric acid. Depending on the cell type, MRP4 may localize to either apical or basolateral membranes in polarized cells. The adaptor protein NHERF1 has previously been implicated in MRP4 internalization in non-polarized cells. We have now found that NHERF1 levels are very low in polarized MDCKI cells which express MRP4 on basolateral membranes relative to polarized LLC-PK1 cells which express MRP4 on apical membranes. Furthermore, ectopic expression of FLAG-tagged NHERF1 in MDCKI cells and in MDCKI cells stably expressing eGFP-tagged MRP4 causes endogenous MRP4 and eGFP-MRP4, respectively, to traffic to the apical membranes. These data establish NHERF1 as a major determinant of MRP4 trafficking to apical membranes of mammalian kidney cells.

Multidrug resistance protein 4 (MRP4) belongs to subfamily C (also known as ABCC) of the ATP-binding cassette superfamily of membrane transport proteins. The sequence identity of ABCC family members ranges from 31% to 58% and many of these proteins are efflux pumps that bind and hydrolyse ATP to drive the translocation of their substrates across membranes [1]. MRP4 (ABCC4) is the smallest of the ABCC proteins with just 1325 amino acids [2,3]. It is widely expressed in a range of tissues [4–6] and blood cell types including erythrocytes and myeloid progenitors [7,8]. The substrate specificity of MRP4 is very broad and only partially overlaps with that of the other MRP-related transporters. Thus, MRP4 transports many important therapeutic agents as well as a variety of endogenous molecules that have key roles in cell signaling pathways [4]. For example, MRP4 confers resistance to nucleobase analogs such as 6-mercaptopurine, nucleotide analogs such as the antiviral 9-[2-(phosphonylmethoxy)ethyl]-adenine (PMEA) and nucleoside analogs such as ganciclovir [4–6,9]. Cyclic nucleotides (e.g. cAMP), eicosanoids (e.g. prostaglandins E_1 and E_2), conjugated steroids (e.g. estradiol glucuronide) and uric acid as well as certain bile acids are also transported by MRP4 [4–6,10,11]. Studies in *Mrp4 (Abcc4)* knockout mice have confirmed that MRP4 plays a protective role with respect to the toxicity of topotecan and thiopurines as well as the disposition of several antibiotics and antiviral agents [7,12,13–15]. They also support a physiological role for MRP4 in cAMP-mediated signaling and nociceptive responses through its ability to mediate transport of cAMP and prostaglandin E_2 [16].

In addition to having a unique substrate specificity, MRP4 distinguishes itself from other ABCC subfamily members by its dual membrane localization in polarized cell types. For example, in prostate tubuloacinar cells, hepatocytes, and choroid plexus epithelium, MRP4 is localized to the basolateral membrane, whereas in renal proximal tubules and the luminal side of brain capillary epithelium it is expressed at the apical membrane [4,17].

The COOH-terminus of MRP4 contains a consensus class I PDZ-interaction motif that is quite similar to that found in MRP2 (ABCC2) and CFTR (ABCC7), both of which localize to the apical membranes of polarized cells [18–21]. In all three ABCC proteins, their PDZ motifs have been demonstrated to be important for their association with the adaptor protein Na^+/H^+ exchanger regulatory factor 1 (NHERF1) [20,22]. For CFTR, NHERF1 enhances the cell surface expression of this chloride channel by increasing its recycling through endosomes [22]. In contrast, NHERF1 is involved in MRP4 internalization, an early step of protein trafficking in non-polarized cells [21]. Thus, downregulation of NHERF1 in HeLa cells causes an increase in MRP4 plasma membrane expression concomitant with an increase in MRP4-mediated drug efflux activity. In the present study, we have investigated the possibility that NHERF1 is also involved in MRP4 trafficking in polarized cells using two kidney cell lines known to differ in their membrane protein sorting properties: LLC-PK1 and MDCKI [23–25].

* Corresponding author. Fax: +1 613 533 6830.
E-mail address: spc.cole@queensu.ca (S.P.C. Cole).

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Materials and methods

Expression vectors. Expression vectors encoding human MRP4 in pcDNA3.1 (pcDNA-MRP4), and peGFP-C3 (peGFP-MRP4) with enhanced green fluorescent protein (eGFP) fused to the NH_2-terminus of the transporter have been described previously [21]. FLAG-tagged rat NHERF1 in p3XFLAG-CMV-7 was provided by Dr. B. Doctor (University of Colorado, Denver, CO) [26].

Preparation of membrane vesicles and estradiol glucuronide ($E_2$17βG) transport assays. The pcDNA3.1 vectors containing wild-type MRP4 cDNA (MRP4) and eGFP-tagged wild-type MRP4 cDNA (eGFP-MRP4) were transfected into HEK293T cells and then inside-out membrane vesicles prepared for organic anion transport assays [27]. Vesicles from untransfected cells served as negative controls. Levels of MRP4 and eGFP-MRP4 in the membrane vesicles were determined by immunoblotting (see below) [21]. ATP-dependent uptake of ^{3}H-labeled $E_2$17βG (45 Ci mmol^{-1}) (PerkinElmer Life Sciences) by the membrane vesicles was measured using a rapid filtration technique also as described previously [27]. Transport assays were carried out in triplicate and results expressed as means (±SD).

Stable transfections of polarized kidney cell lines. The polarized kidney cell lines MDCKI and LLC-PK1 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum. MDCKI and LLC-PK1 cells were transfected with the peGFP-MRP4 expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and incubated in G418 (Geneticin) (GIBCO) beginning 2 days after transfection. After 2–3 weeks, several G418-resistant colonies were screened by immunoblotting (see below) and fluorescence-activated cell sorting using an EPICS ALTRA HSS instrument. Single MDCKI and LLC-PK1 clones with the highest levels of eGFP-MRP4 expression were selected for further studies.

SDS–PAGE and immunoblotting. Immunoblotting was performed as described [21]. The primary antibodies used for immunoblotting were as follows: mouse monoclonal antibody (mAb) raised against NHERF1 (EBP-10, 1 mg ml^{-1}) (Alexis Biochemicals); rat mAb against MRP4 (M_4I-10) (~150 μg ml^{-1}) (Alexis Biochemicals) [12]; and mouse mAbs against GFP (Chemicon), α-tubulin (2 mg ml^{-1}) (Sigma), and FLAG (1.0 mg ml^{-1}) (Sigma) (all diluted 1:3000). The horseradish peroxidase-conjugated anti-rat, anti-mouse and anti-rabbit (all from Pierce), and anti-goat (Santa Cruz Biotechnology) secondary antibodies were used at a dilution of 1:10,000.

Fluorescence microscopy. In initial experiments, LLC-PK1 and MDCKI cells expressing eGFP-MRP4 were either plated on coverslips coated with poly-L-lysine or on 0.4 μM transwell polycarbonate filters (Corning Coster Inc., Cambridge, MA) and then allowed to establish confluency over >3 days. Filters were then removed, fixed in cold ethanol, and mounted on glass slide. Images were then collected using a Leica TCS SP2 multiphoton confocal microscope equipped with a PL APO 100X/1.40 plan oil immersion lens (Leica). Subsequent immunofluorescence experiments with anti-FLAG and anti-MRP4 antibodies were performed essentially as described [21,28]. MDCKI cells and cells stably overexpressing eGFP-MRP4 protein transfected with FLAG-tagged NHERF1 cDNA were plated on transwell polycarbonate filters and confluent cells cultured for a further 3 days. Filters were removed, fixed, and analyzed by indirect immunofluorescence using mouse anti-FLAG mAb and rat anti-MRP4 mAb. Images were collected at an 8-bit depth and 1024 × 1024 pixel resolution using LCS software (Leica). Cross-sections were generated with a 0.2 μm motor step.

Results

Addition of an eGFP tag to MRP4 does not affect its transport activity

Previous studies have shown that MRP4 localization in polarized cells varies depending on the cell type in which it is expressed. For example, in renal proximal tubule cells it is expressed in apical membranes whereas in prostate tubulocinar cells, it is found in basolateral membranes [2]. Prior to determining the membrane localization of ectopically expressed eGFP-tagged MRP4 in the MDCKI and LLC-PK1 cell lines, it was necessary to establish that the addition of an eGFP tag to the NH_2-terminus of MRP4 did not significantly affect its expression or activity. Thus, HEK293T cells were transiently transfected with either untagged MRP4, eGFP-tagged MRP4 cDNAs, or not transfected (control), and inside-out membrane vesicles were prepared, levels of MRP4 confirmed by immunoblotting and then activity compared using a vesicular transport assay. As shown in Fig. 1A, the HEK293T cells expressed similar levels of the tagged and untagged MRP4 proteins. The two proteins also exhibited very similar time-dependent rates of ATP-dependent [^{3}H]$E_2$17βG vesicular uptake (Fig. 1B). At 5 min, the [^{3}H]$E_2$17βG uptake levels for MRP4 and eGFP-MRP4 were 211 ± 24 and 199 ± 16 pmol mg protein^{-1}, respectively.

eGFP-MRP4 localizes to the basolateral and apical membranes of MDCKI and LLC-PK1 cells, respectively

We next examined the localization of eGFP-tagged MRP4 in the polarized MDCKI and LLC-PK1 cell lines which are believed to originate from kidney distal and proximal tubules, respectively [29,30]. To do this, eGFP-MRP4 cDNA was first stably transfected into MDCKI and LLC-PK1 cells, and clonal eGFP-MRP4 expressing cell lines isolated by fluorescence-activated cell sorting. As shown in

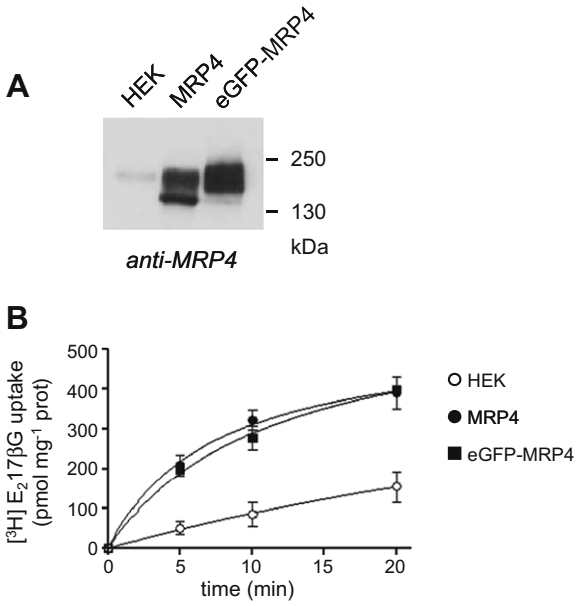

Fig. 1. Expression and [^{3}H]$E_2$17βG uptake activity of MRP4 and eGFP-MRP4. (A) Membrane vesicles (1 μg protein) prepared from untransfected HEK293T cells (HEK) or cells transfected with untagged (MRP4) or eGFP-tagged (eGFP-MRP4) wild-type MRP4 expression vectors were immunoblotted with anti-MRP4 mAb M_4I-10 [12]. (B) ATP-dependent uptake of [^{3}H]$E_2$17βG (400 nM) in membrane vesicles prepared from HEK293T cells expressing MRP4 (●) and eGFP-MRP4 (■) was measured at 37 °C for the times indicated. Uptake by vesicles prepared from untransfected cells was measured as a control (○). The results shown are means (±SD) of triplicate determinations in a single experiment.

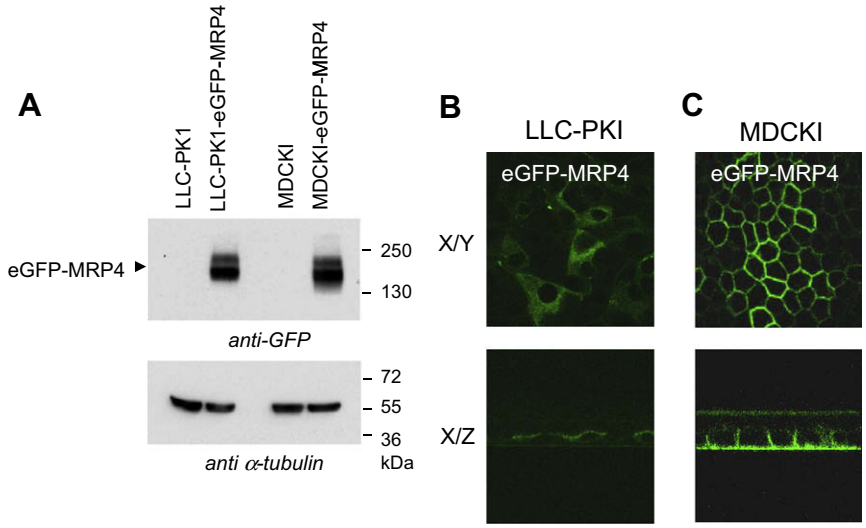

Fig. 2. Membrane localization of eGFP-MRP4 in transfected MDCKI and LLC-PK1 cells. (A) Whole cell lysates (30 μg protein per lane) prepared from LLC-PK1 and MDCKI cells stably expressing eGFP-MRP4 were immunoblotted using anti-GFP antibody. Lysates prepared from untransfected cells were used as controls. Blots were also probed with anti-α-tubulin antibody to verify equal loading of the wells. (B,C) LLC-PK1 cells (B) and MDCKI cells (C) were grown on coverslips, fixed and then analyzed for eGFP fluorescence. eGFP top panels, X/Y (parallel section); bottom panels, X/Z (cross-sections).

Fig. 2A, eGFP-MRP4 could be readily detected in both the transfected MDCKI and LLC-PK1 cell lines by immunoblotting with an anti-eGFP antibody. The transfected cell lines were then analyzed by confocal microscopy after growing cells as confluent monolayers on polycarbonate filters. As shown in Fig. 2C, eGFP-MRP4 was localized predominantly to the apical membranes in LLC-PK1 cells but was restricted to basolateral membranes in MDCKI cells (Fig. 2B). It should be noted that there are two variants of MDCK cells; the first, MDCKI, is derived from an early passage of the cell line while the second, MDCKII, is derived from a later passage [23]. The two variants are reported to differ substantially in their glycosphingolipid content and their transepithelial electrical resistance [23]. However, it appears that in both variant cell lines, MRP4 is localized on the basolateral membrane (Fig. 2C; [31]).

In non-polarized HeLa cells, we have recently described a role for the adaptor protein NHERF1 in MRP4 internalization, an early step of protein trafficking [21]. It was therefore of interest to investigate whether NHERF1 might have a role in MRP4 trafficking to the apical/basolateral membranes of the polarized kidney cell lines used here. Initially, the levels of endogenous NHERF1 in MDCKI and LLC-PK1 cells were determined by immunoblotting of whole cell lysates. As shown in Fig. 3A, NHERF1 was readily detectable in the LLC-PK1 cells but not in the MDCKI cells, even when blots were exposed to film for up to 10 min (not shown). Furthermore, as shown in Fig. 3B (bottom panel), endogenous MRP4 was detectable in lysates prepared from both cell lines although the relative levels of the transporter appeared substantially greater in MDCKI cells than in LLC-PK1 cells. Together, these results indicate that although MRP4 expression is detectable in the membranes of both kidney cell lines, it is apically located only in LLC-PK1 cells which express substantial levels of NHERF1.

Ectopic expression of NHERF1 promotes MRP4 localization to the apical membranes of MDCKI cell

The differential expression of NHERF1 suggested that its expression in LLC-PK1 cells might be involved in apical localization of MRP4. To investigate this possibility, a FLAG-tagged NHERF1 cDNA expression vector was first transfected into MDCKI cells (which express no detectable endogenous NHERF1). Immunoblots revealed a band at 50–60 kDa (a size corresponding to that predicted for the FLAG-tagged NHERF1 protein) in the transfected cells but not in the untransfected cells as expected (Fig. 4A). Confluent monolayers of FLAG-NHERF1 transfected MDCKI cells were then cultured for an additional three days to facilitate polarization, and then fixed and analyzed by confocal microscopy using anti-MRP4 and anti-FLAG mAbs. As shown in Fig. 4B, endogenous MRP4 was restricted to basolateral membranes in the untransfected MDCKI cells while in MDCKI cells overexpressing FLAG-NHERF1, MRP4 localized to apical membranes. Similarly, when FLAG-NHERF1 cDNA was transfected into MDCKI cells overexpressing eGFP-MRP4, eGFP-MRP4 was also found on the apical membranes in contrast to untransfected cells lacking NHERF1 expression where eGFP-MRP4 was found on basolateral membranes (data not shown). Taken together, these results indicate that ectopic expression of NHERF1 can redirect trafficking of MRP4 to apical membranes in MDCKI cells.

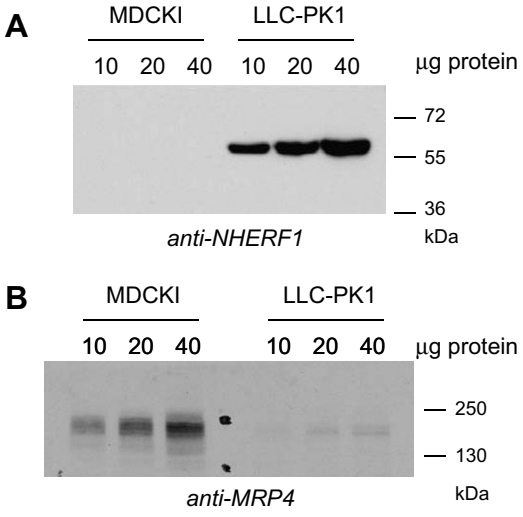

Fig. 3. Expression of endogenous NHERF1 and MRP4 in intact LLC-PK1 and MDCKI cells. Lysates were prepared from MDCKI and LLC-PK1 cells, proteins separated by SDS–PAGE (10, 20, and 40 μg protein per lane) and then immunoblotted using (A) anti-NHERF1, and (B) anti-MRP4 antibodies, as indicated.

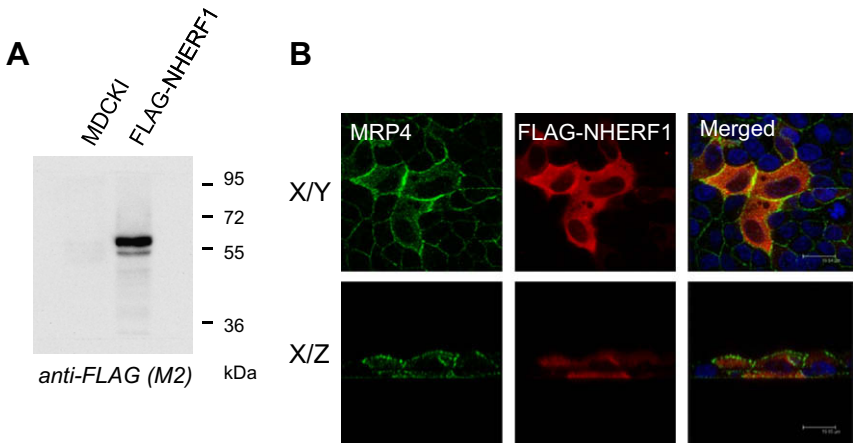

Fig. 4. Ectopic expression of NHERF1 promotes localization of MRP4 to the apical membranes of MDCKI cells. (A) Whole cell lysates (30 μg protein per lane) were prepared from untransfected MDCKI control cells or MDCKI cells transfected with FLAG-tagged NHERF1 and immunoblotted using an anti-FLAG antibody. (B) Confluent MDCKI cells transfected with a FLAG-NHERF1 cDNA expression vector were fixed in ethanol and analyzed by indirect immunofluorescence using anti-FLAG (red) and anti-MRP4 (green) antibodies. Scale bars, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Discussion

Despite their substantial levels of sequence identity, the ABCC-related proteins are not all expressed on the same membranes in polarized cells. Thus, while MRP2 and CFTR localize to apical membranes, the remainder typically localize to basolateral membranes. The exception is MRP4 which can localize to either membrane depending on the cell type in which it is expressed. While the physiological and pharmacological implications of this dual localization are far from fully understood, there is currently considerable evidence to support the idea that the apical membrane localization of MRP4 in the kidney proximal tubules plays an important role in the excretion of a number of important therapeutic agents as well as for maintaining urate homeostasis in the blood [13,14,32–34]. It is therefore important to understand the mechanisms and molecules that determine membrane trafficking of this efflux transporter.

Here we have demonstrated the differential localization of MRP4 to apical and basolateral membranes in the mammalian kidney cell lines LLC-PK1 and MDCKI, respectively. We have also found that LLC-PK1 cells contain a substantial amount of NHERF1 which we have recently demonstrated interacts with the COOH-terminal PDZ-motif ETAL in MRP4 at least in non-polarized human HeLa and HEK293T cells [21]. Further, when NHERF1 was ectopically expressed in MDCKI cells (which express very little endogenous NHERF1), we observed that MRP4 trafficking was redirected from the basolateral to apical membranes. Together, these observations provide strong evidence that NHERF1 is involved in apical targeting of this transporter. It is of interest that the apically located urate uptake transporter, URAT1, which like MRP4 is located in the renal proximal tubules also interacts with NHERF1 [35]. Thus, it may be that in this region of the kidney, NHERF1 serves an important role in urate homeostasis by co-regulating urate uptake and efflux through its interactions with URAT1 and MRP4, respectively.

Our present findings are of interest with respect to those reported earlier for the related CFTR (ABCC7) where the PDZ-motif in this chloride channel to which NHERF1 binds has been demonstrated to be important for its apical localization in both human airway and kidney epithelial cells [36]. However, in striking contrast to MRP4, CFTR is also found on apical membranes in MDCKI cells which express little NHERF1 [37]. It may be that apical localization of CFTR is directed by a different adaptor protein in MDCK cells (e.g. such as NHERF2 [38]) but even if this is the case, such an alternate adaptor protein clearly does not interact with MRP4 in the same way. Thus, our present observations of differing interactions of MRP4 and CFTR with NHERF1 in polarized kidney cells are consistent with our previous findings in non-polarized cells demonstrating the contrasting role of NHERF1 in MRP4 internalization versus its role in enhancing cell surface expression of CFTR by increasing recycling of this chloride channel through endosomes [21,22].

Similar to our findings here with MRP4, the β-subunit of H,K-ATPase has also been reported to localize in basolateral and apical membranes in MDCK and LLC-PK1 cells, respectively [24,39]. The H,K-ATPase β-subunit contains a tyrosine-based basolateral signal that is required for its binding to μ1B, a component of the clathrin adaptor protein 1 (AP-1) complex, and it is this complex which directs sorting of this ATPase to basolateral membranes in MDCK cells [24]. However, in contrast to the redirecting of MRP4 trafficking to the apical membrane in MDCKI cells by ectopically expressed NHERF1 as we have demonstrated here, the membrane sorting of the H,K-ATPase β-subunit is not redirected to the basolateral membrane of LLC-PK1 cells by ectopic expression of μ1B. Therefore, it has been proposed that the apical sorting of the H,K-ATPase β-subunit in LLC-PK1 cells is due to the presence of active apical sorting information in this protein that can override the effects of μ1B expression, such as some sort of clathrin-independent pathway [39].

At present it is not clear what directs basolateral membrane targeting of MRP4 in polarized MDCKI cells or in certain tissues in the body such as prostate tubuloacinar cells or hepatocytes [3,10]. Examination of the amino acid sequence of MRP4 reveals the presence of a putative tyrosine-based basolateral signal motif at position 1255 but whether or not this motif interacts with μ1B or other adaptor protein to direct its sorting to the basolateral plasma membrane remains to be determined.

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