Down-regulation of Na\textsuperscript{+}/H\textsuperscript{+} Exchanger Regulatory Factor 1 Increases Expression and Function of Multidrug Resistance Protein 4

Md. Tozammel Hoque\textsuperscript{1,2} and Susan P.C. Cole\textsuperscript{1}

\textsuperscript{1}Division of Cancer Biology and Genetics, Cancer Research Institute, Queen’s University, Kingston, Ontario, Canada; and
\textsuperscript{2}Institute of Biological Sciences, Rajshahi University, Rajshahi, Bangladesh

Abstract
Multidrug resistance protein 4 (MRP4; ABCC4) is a member of the ATP-binding cassette superfamily of membrane transport proteins and confers resistance to nucleoside and nucleotide analogues as well as camptothecin derivatives. MRP4 also mediates the transmembrane transport of several eicosanoids, conjugated estrogens, and cyclic AMP. The subcellular localization of MRP4 depends on the cell type in which it is expressed, but the molecular determinants responsible for trafficking of MRP4 to the plasma membrane are unknown. Here, we describe the interaction of Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor 1 (NHERF1) with MRP4 via the last four amino acids (\textsuperscript{1322}ETAL\textsuperscript{1325}) of the transporter. Down-regulation of NHERF1 by small interfering RNA (siRNA) in HeLa cells significantly increased MRP4 levels at the plasma membrane, suggesting that internalization of the transporter was inhibited. Increased plasma membrane MRP4 was accompanied by increased efflux function as reflected by reduced cellular accumulation of the MRP4 substrates 6-mercaptopurine and 9-[2-(phosphonomethoxy)ethyl]-adenine. Furthermore, enhanced green fluorescent protein-tagged MRP4 was internalized in monensin-treated cells, and this internalization was markedly reduced after NHERF1 down-regulation by siRNA. Together, these data establish NHERF1 as a novel protein-binding partner of MRP4 that plays a significant role in the internalization and drug efflux function of this transporter.

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Introduction
ATP-binding cassette (ABC) transporters comprise a large superfamily of integral membrane proteins, the vast majority of which mediate the translocation of solutes across biological membranes. The multidrug resistance proteins (MRP) belong to subfamily C (also referred to as ABCC) of the ABC superfamily, which contains 13 genes designated \textit{ABCC} through \textit{ABCC13}. The three best-characterized ABC transporters, MRP1, MRP2, and MRP3 (\textit{ABCC}1, \textit{ABCC}2, and \textit{ABCC}3), are known to transport a broad range of chemically diverse xenobiotic conjugates and other organic anions and are characterized by a five-domain structure with three membrane-spanning domains (MSD) and two nucleotide-binding domains. In contrast, MRP4 (\textit{ABCC}4), MRP5 (\textit{ABCC}5), MRP8 (\textit{ABCC}11), and MRP9 (\textit{ABCC}12) lack the NH\textsubscript{2}-terminal MSD of MRP1, MRP2, and MRP3 and thus have a more typical four-domain ABC structure (1, 2).

The gene coding for MRP4 is located on the long arm of chromosome 13 at band q32 (3, 4). The MRP4 mRNA transcript encodes the smallest of the MRP-related proteins with just 1,325 amino acids. Expression of human \textit{MRP4}/\textit{ABCC4} was initially reported to be restricted to a few tissues. However, subsequent studies have shown that it is widely expressed with MRP4 mRNA levels ranging from very high in prostate to barely detectable in liver. Furthermore, immunohistochemical studies have detected MRP4 protein in prostate, kidney, liver, brain, and platelets as well as in several cell lines (5).

The substrate specificity of MRP4 is very broad and only partially overlaps with MRP1, MRP2, and MRP3. Thus, like MRP1, MRP2, and MRP3, MRP4 transports certain glutathione (GSH) and glucuronide conjugates and sulfated steroids, but unlike MRP1, MRP2, and MRP3, it also transports several cyclic nucleotides, bile acids, and unconjugated eicosanoids as well as uric acid (6–13). MRP4 also confers resistance to nucleobase analogues such as 6-mercaptopurine and 6-thioguanine, nucleotide analogues such as the antiviral 9-[2-(phosphonomethoxy)ethyl]-adenine (PMEA), and nucleoside analogues such as ganciclovir (14–18). MRP4 is also a resistance factor for methotrexate and several camptothecins (8, 15, 19, 20).

The COOH terminus of MRP4 contains a consensus class I PDZ interaction motif (ETAL, X-(S/T)-X-\Phi, X, unspecified; \Phi, hydrophobic residue), which is quite similar to that found in the related proteins MRP2 (\textit{ABCC}2) and CFTR (\textit{ABCC}7), both of which localize to the apical membranes of polarized cells (Fig. 1A; ref. 21). The COOH-terminal PDZ motifs in MRP2 (STKF) and CFTR (DTRL) have been reported to be important for their association with Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor 1 (NHERF1), also known as ezrin-binding protein 50 (22, 23). In the case of CFTR, NHERF1 enhances the cell surface expression of this chloride channel by increasing its recycling, whereas in the case of MRP2 the functional consequences of its interaction with NHERF1 have not yet been determined (23, 24).

In the present study, we have investigated the possibility that NHERF1 also interacts with MRP4. We have found that the COOH terminus of MRP4 directly interacts with NHERF1 in glutathione \textit{S}-transferase (GST) pull-down assays, and this interaction is abrogated by deletion of the last four amino acids of MRP4, thus identifying ETAL as a functional PDZ interaction motif. The association between endogenous MRP4 and NHERF1 was further confirmed in intact HeLa and HEK293T cells. Down-regulation of NHERF1 by small interfering RNA (siRNA) in HeLa cells significantly increased MRP4 protein levels at the plasma membrane, which was associated with an increase in the drug efflux activity of the transporter. The elevated surface expression of MRP4 seems attributable to a substantial decrease in transporter internalization, indicating that NHERF1 is required for MRP4 internalization. Consistent with this idea, enhanced green fluorescent protein (eGFP)-tagged wt-MRP4 was found to internalize in monensin-treated
HeLa cells, and this internalization was dramatically reduced when NHERF1 was down-regulated by siRNA. Our results thus identify NHERF1 as a novel binding partner of MRP4 and point to a crucial although unanticipated role for NHERF1 in MRP4 internalization and drug efflux function.

Materials and Methods

Expression vectors. The pcDNA3.1/Hygro-MRP4 vector encoding the full-length human MRP4 was kindly provided by Dr. Dietrich Keppler (Heidelberg Cancer Centre, Heidelberg, Germany) and subcloned into pcDNA3.1(C0) at the Nhe/1/Xho/I sites to generate pcDNA3.1(C0)-wt-MRP4 (9). To generate a mutant lacking the last four amino acids, pcDNA3.1(C0)-MRP4ΔETAL, a 3¶ fragment encoding amino acids 966 to 1,321 of MRP4 was amplified from pcDNA3.1(−)wt-MRP4 by PCR using Pfu DNA Polymerase (Stratagene) with the following mutagenic primer set (Integrated DNA Technologies): (5¶-TTTTTGGAAACGTCAAGAGATGTGAAGCG-3¶ and 5¶-TTGCTCGAGCTAAGAAAATAGTTAAGGTCGAGGGCTGTCCATTG-3¶), which were designed to place a stop codon (boldface) before the last four amino acids of MRP4 and to create a 3¶Xho/I site (underlined). The 1.1-kb PCR fragment was digested with EcoRI and cloned into pcDNA3.1(C0)-wt-MRP4. To generate eGFP-tagged MRP4 (peGFP-wt-MRP4) and MRP4ΔETAL (peGFP-MRP4ΔETAL) constructs, the wild-type MRP4 cDNA and the mutant MRP4ΔETAL were excised from pcDNA3.1(C0)-wt-MRP4 and pcDNA3.1(C0)-MRP4ΔETAL vectors by EcoRI digestion and cloned into peGFP-C3 (Clontech).

To produce GST fusion proteins with different COOH-terminal truncations, cDNA fragments encoding MRP4 amino acids 1,209 to 1,325 (MRP4-CT) and 1,210 to 1,321 (MRP4-CTΔETAL) were excised from pcDNA3.1(−)wt-MRP4 and cloned into pGEX6P-1 (Amersham Pharma Biotech) at the EcoRI and Xho/I sites to generate GST-MRP4-CT and GST-MRP4-CTΔETAL vectors, respectively.

Figure 1. NHERF1 associates with the COOH terminus of MRP4 in HEK293T and HeLa cells. A, PDZ consensus X-(S/T)-X-A (where A is a hydrophobic amino acid and X is any amino acid) and the last four amino acids of MRP4, CFTR, and MRP2. B, recombinant GST-MRP4-CT, GST-MRP4-CTΔETAL, or GST alone was immobilized on GSH-Sepharose 4B and used to pull down NHERF1 from HEK293T (top) and HeLa (bottom) cell lysates. The bound fractions were separated on 10% SDS-PAGE and immunoblotted (IB) with anti-NHERF1 antibody. For each cell lysate, immunoblots are shown on the left, whereas amido black staining of the membrane is shown on the right. C and D, reciprocal coimmunoprecipitation of NHERF1 and eGFP-tagged MRP4. Cell lysates prepared from HEK293T cells transfected with either eGFP-wt-MRP4 or eGFP-MRP4ΔETAL were precleared with normal rabbit immunoglobulin and then subjected to immunoprecipitation with rabbit polyclonal anti-GFP antibody. C, the immunoprecipitates were separated on a 4% to 20% polyacrylamide gradient gel and first immunoblotted with mouse monoclonal anti-NHERF1 antibody and detected by horseradish peroxidase (HRP)-conjugated anti-rabbit antibody. D, the membrane was stripped and again immunoblotted with rabbit polyclonal anti-GFP antibody and detected with HRP-conjugated anti-rabbit antibody (Pierce). Lanes 1 and 5, input; lanes 2 and 6, normal rabbit IgG; lanes 3 and 7, anti-GFP immunoprecipitation; lane 4, molecular weight markers; lanes 1 to 3, lysate from eGFP-wt-MRP4-expressing cells; lanes 5 to 7, lysate from eGFP-MRP4ΔETAL-expressing cells. Molecular weight standards are marked in kDa.
pcDNA3.1(−)-wt-MRP4 and pcDNA3.1(−)-MRP4ΔETAL, respectively, by BamHI/XhoI digestion and subcloned into pGEX-6P-2 (Amersham Biosciences).

Cell culture and transfections. HEK293T and HeLa cells were cultured in DMEM/10% fetal bovine serum and transfected at 50% to 80% confluence using Lipofectamine 2000 (Invitrogen) and grown for 48 h at 37°C before harvesting.

SDS-PAGE and immunoblotting. Immunoblotting was performed as described (25). The primary antibodies used for immunoblotting and immunofluorescence (at 1:1,000 and 1:400 dilutions, respectively) were as follows: rabbit anti-MRP4 (1.5–2.0 mg/mL), mouse monoclonal antibody (mAb) against NHERF1 (1 mg/mL), and rat mAb against MRP4 (M4I-10; Santa Cruz Biotechnology). Anti-GFP (FL) antibody was used at a 1:500 dilution (200 mg/mL; Santa Cruz Biotechnology).

Down-regulation of NHERF1 by siRNA. The siRNA used to knock down the NHERF1 mRNA was 5'-GGAGAACAGUCGUAAGCCTT-3', designed according to the manufacturer's instructions and synthesized as annealed siRNAs of standard purity (Ambion). HeLa cells were transfected with either control (control #1; Ambion) or NHERF1 siRNAs as described (26).

Drug accumulation assay. Drug accumulation experiments were performed as described with few modifications (17). Briefly, 2.5 × 10^5 cells per well were seeded in triplicate in a six-well plate and, 24 h later, washed and incubated with 0.25 μCi/mL of the PMEA prodrug [adenine-8-^3H] bis(pivaloyloxy)methyl-9-[2-((phosphonomethoxy)ethy1)-adenine [bis(POM)PMEA; 10.7 Ci/mmol] or 0.5 μCi/mL of [8-^14C]6-mercaptopurine (51 mCi/mmol) obtained from Moravek Biochemicals. After the desired time, cell monolayers were washed with PBS, trypsinized, and resuspended in PBS, and radioactivity of a small aliquot was determined by liquid scintillation counting.

Indirect immunofluorescence analyses. Immunofluorescence experiments were performed as described (27) and fluorescence images were collected using Leica TCS SP2 multiphoton confocal microscope equipped with a PL APO 100×/1.40 plan oil immersion lens (Leica). Images were collected at an 8-bit depth and 1,024 × 1,024 pixel resolution using LCS software (Leica).

Expression and purification of recombinant proteins in bacteria. GST-tagged MRP4-CT and MRP4-CTΔETAL fusion proteins were produced by transforming BL21(DE3)-RIL Escherichia coli cells (Stratagene) with pGEX-6P-2-MRP4-CT or pGEX-6P-2-MRP4-ΔETAL and incubating the cells with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 to 4 h at 30°C. Expressed proteins were purified as described (28), and after dialyzing against PBS (pH 7.4), their integrity was confirmed by SDS-PAGE and Coomassie blue staining.

Results and Discussion

Identification of NHERF1 as a binding partner of MRP4. The role of MRP2 (ABCC2) and CFTR (ABCC7) PDZ motifs in mediating interaction with NHERF1 has been reported previously (22, 23). MRP4 possesses a COOH-terminal sequence that fits a PDZ domain consensus similar to that of MRP2 and CFTR (Fig. 1A, ref. 21), and therefore, we investigated the possibility that MRP4, like MRP2 and CFTR, might also associate with NHERF1. In initial experiments, a fusion protein pull-down approach was used. Two recombinant proteins encoding the COOH-terminal 117 (1,209–1,325 amino acids, GST-MRP4-CT) and 112 (1,210–1,321 amino acids, GST-MRP4-CTΔETAL) amino acids of MRP4 were expressed and purified as GST fusion proteins. Equal amounts of these fusion proteins were then adsorbed onto GST-Sepharose beads and

Figure 2. Coimmunoprecipitation and colocalization of endogenous MRP4 and NHERF1 in HeLa and HEK293T cells. A, representative immunoblots. Cell lysates (input) were prepared from exponentially growing HeLa cells and subsequently precleared by immunoprecipitation (IP) with normal mouse immunoglobulin (NMI). Precleared lysate was then immunoprecipitated with mouse anti-NHERF1 mAb. Top, both NMI and NHERF1 immunoprecipitates were separated by 7% SDS-PAGE and first blotted with rat anti-MRP4 mAb; bottom, the same membrane was stripped and reprobed with mouse anti-NHERF1 mAb. B, PNGase F–treated (+) or untreated (−) HeLa lysates (20 μg protein per lane) were immunoblotted with rat anti-MRP4 mAb to visualize MRP4 deglycosylation. C, asynchronous HEK293T cells were fixed and DNA was stained with 4',6-diamidino-2-phenylindole (DAPI; blue) and examined by indirect immunofluorescence with rat anti-MRP4 mAb (red) and mouse anti-NHERF1 mAb (green) as described in Materials and Methods. Colocalization of MRP4 and NHERF1 on the plasma membrane is indicated by a yellow color in the merged image. Bar, 25 μm.
incubated with HEK293T cell extracts. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-NHERF1 antibody.

As shown in Fig. 1B (top half), GST-MRP4-CT, which contains the putative PDZ interaction motif, efficiently pulled down NHERF1 (lane 3). In contrast, GST-MRP4-CTΔETAL and GST alone did not detectably bind to NHERF1 in the cell lysates under the same conditions (lanes 2 and 4). Comparable results were obtained when pull-down experiments were performed with HeLa cell extracts (Fig. 1B, bottom half). Thus, GST-MRP4-CT, but not MRP4-CTΔETAL, can associate with endogenous NHERF1 in both HEK293T and HeLa cells. This interaction between MRP4 and NHERF1 is expected to occur in other species because the ETAL sequence of human MRP4 is present in the MRP4 protein sequences of all other species known to date (mouse, rat, dog, and cow).

**MRP4 associates with NHERF1 in intact cells.** We next wished to determine if full-length MRP4 also associates with NHERF1 in a cellular context. Thus, HEK293T cells were transiently transfected with eGFP-wt-MRP4 or eGFP-MRP4ΔETAL recombinant constructs, cell lysates were prepared, and immunoprecipitations were performed with an anti-GFP antibody. After immunoblotting with anti-NHERF1 antibody, severalfold higher amounts of NHERF1 were observed in coimmunoprecipitates of lysates prepared from eGFP-wt-MRP4–expressing cells than with lysates from cells expressing eGFP-MRP4ΔETAL (Fig. 1C, lane 3 versus lane 7). Immunoblotting of the same membrane with an anti-GFP antibody confirmed that equal amounts of recombinant eGFP-wt-MRP4 and eGFP-MRP4ΔETAL proteins were immunoprecipitated (Fig. 1D, lanes 3 and 7). These data confirmed the specificity of the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effect of depleting NHERF1 on endogenous MRP4 expression and drug accumulation in HeLa cells. A, equal protein loadings of homogenates derived from either control siRNA–treated HeLa cells or HeLa cells treated with siRNA directed against NHERF1 were subjected to immunoblotting. The membranes were probed with anti-NHERF1 antibody and subsequently stripped and probed with anti-MRP4 or anti-α-tubulin antibodies (loading control). NHERF1 was undetectable after siRNA knockdown (top), whereas the MRP4 signal was enhanced (>50% of control) under the same conditions (middle). B, confocal images of HeLa cells treated with either control siRNA (top) or siRNA directed against NHERF1 (bottom). Cellular DNA was stained with DAPI (blue) and cells were analyzed by immunofluorescence using anti-MRP4 (red) and anti-NHERF1 (green) antibodies. Colocalization of MRP4 and NHERF1 is indicated by a yellow color in the merged image. Note that MRP4 labeling associated with the plasma membrane signal was markedly increased in NHERF1 siRNA–treated cells (bottom) relative to control siRNA–treated cells (top). Bars, 20 μm. C, accumulation of [3H]PMEA in control (white columns) and NHERF1 siRNA–treated (black columns) HeLa cells. Cells were incubated with 25 nmol/L [3H]bis(POM)PMEA at 37°C and cell-associated radioactivity was measured at the indicated time points.
interactions between NHERF1 and MRP4 via the four COOH-terminal amino acids of the transport protein. Thus, our studies add MRP4 to the list of ABC/MRP subfamily members having a COOH-terminal PDZ interaction motif that interacts with NHERF1.

To determine whether endogenous MRP4 and NHERF1 physically interact in intact cells, HeLa cell extracts were prepared and immunoprecipitated with an anti-NHERF1 antibody and then immunoblotted with a rat anti-MRP4 mAb (Fig. 2C, top). The same membrane was stripped and reprobed with an anti-NHERF1 antibody (Fig. 2A, bottom). These experiments revealed the coimmunoprecipitation of a protein complex containing endogenous MRP4 and NHERF1 (Fig. 2A, top and bottom). In addition, when similar immunoprecipitation and immunoblotting experiments were performed with HEK293T cell extracts, comparable results were obtained (data not shown). The MRP4 mAb detected two bands with electrophoretic mobilities of approximately 160 and 180 kDa. The upper band is most likely a glycosylated form of MRP4 because treatment with peptide-N-glycosidase F (PNGase F) reduced its size to 160 kDa (Fig. 2A, top).

We next investigated the colocalization of MRP4 and NHERF1 in HEK293T cells by confocal microscopy using a rat anti-MRP4 mAb and a mouse anti-NHERF1 mAb. Significant amounts of MRP4 and NHERF1 were found to colocalize on the plasma membrane (Fig. 2C). Both proteins also localized to some degree on internal structures. These observations were specific because cells immunostained without primary antibodies did not produce any fluorescent signals (data not shown).

Depletion of NHERF1 from HeLa cells by RNA interference increases MRP4 expression levels. To better understand the role of MRP4-associated NHERF1, NHERF1 was depleted in HeLa cells using siRNA. We found that our siRNA directed against NHERF1 completely eliminated expression of the NHERF1 protein in HeLa cells after 96 h, and the most effective way to deplete NHERF1 was to transfect the cells twice with a 48-h interval between transfections. By phase-contrast microscopy, the NHERF1 siRNA–treated cells seemed healthy (data not shown). As shown in Fig. 3A (top), NHERF1 protein was undetectable by immunoblotting after knockdown with NHERF1 siRNA, whereas a strong signal was detected in control siRNA–treated cells. Furthermore, in HeLa cells where NHERF1 expression was completely abrogated, the levels of MRP4 protein were significantly increased (~3-fold; Fig. 3A, middle).

We also investigated the localization of MRP4 in NHERF1-depleted HeLa cells by double immunofluorescence labeling. In control siRNA-transfected cells, most of the MRP4 and NHERF1 proteins were colocalized in internal structures (yellow signal), although small amounts of both proteins were also detected on the plasma membrane (Fig. 3B, top). In contrast, MRP4 staining was brighter and mostly associated with the plasma membrane in NHERF1 siRNA-transfected cells (Fig. 3B, bottom). These results are consistent with the immunoblot analysis (Fig. 3A).

To better understand if the elevated levels of MRP4 protein at the plasma membrane observed in NHERF1 down-regulated HeLa cells were associated with increased MRP4 function, cellular accumulation of the MRP4 substrates [3H]bis(POM)PMEA and [14C]6-mercaptopurine was determined (7, 17). As shown in Fig. 3C, cellular accumulation of [3H]bis(POM)PMEA and [14C]6-mercaptopurine (data not shown) was significantly reduced in HeLa cells

![Figure 4](image-url)
treated with NHERF1 siRNA compared with control siRNA–treated cells after both 6 and 20 h of incubations with the drugs. These observations indicate that the higher level of MRP4 expression at the plasma membrane that accompanies down-regulation of NHERF1 is associated with greater drug efflux activity resulting in lower levels of drug accumulation in the cells. The lower levels of drug accumulation at 20 h versus 6 h in Fig. 3C are presumably due to the efflux activity of endogenous MRP4.

**eGFP-wt-MRP4 trafficking between the plasma membrane and endocytic compartments.** Monensin is a Na+ ionophore that disrupts intracellular Na+ and H+ gradients leading to retention of internalized membrane proteins in intracellular vesicles because their recycling to the plasma membrane is inhibited (30). Thus, monensin treatment would be expected to reduce surface expression of eGFP-wt-MRP4. In addition, because monensin blocks protein degradation and trafficking out of late endosomes and lysosomes, eGFP-wt-MRP4 would be expected to accumulate in LAMP-2(+) late endosomes and lysosomes (30).

As shown in Fig. 4A (bottom), monensin treatment reduced the level of eGFP-wt-MRP4 residing at the cell surface and enhanced localization of eGFP-wt-MRP4 in intracellular structures containing LAMP-2 as anticipated. In contrast, in the absence of monensin, most of the eGFP-wt-MRP4 remained on the plasma membrane and only a small amount was associated with intracellular structures (Fig. 4A, top). Confocal analyses with the Golgi-specific anti-TGN46 antibody showed no overlap of the eGFP and TGN46 signals in either the presence or absence of monensin, indicating that eGFP-wt-MRP4 is not retained in the Golgi (Fig. 4B). Taken together, these results suggest that monensin blocks the movement of newly synthesized eGFP-wt-MRP4 to the cell surface and traps plasma membrane–derived eGFP-wt-MRP4 in late endocytic compartments.

**NHERF1 down-regulation inhibits MRP4 internalization in HeLa cells.** To further investigate the role NHERF1 plays in MRP4 internalization, we next examined MRP4 internalization in NHERF1 down-regulated HeLa cells. Thus, after first treating...
HeLa cells with NHERF1 siRNA, eGFP-wt-MRP4 was ectopically expressed and cells were exposed to monensin. Cells were also immunostained with anti-LAMP-2 antibodies to visualize endogenous LAMP-2.

As shown in Fig. 5A (bottom), exposure of control siRNA–treated cells to monensin reduced the level of eGFP-wt-MRP4 surface expression and increased the localization of eGFP-wt-MRP4 in intracellular structures containing LAMP-2. In the absence of monensin, however, the majority of the eGFP-wt-MRP4 remained on the cell surface (Fig. 5A, top). In contrast, monensin addition did not change eGFP-wt-MRP4 surface expression in the NHERF1-depleted cells (Fig. 5B, bottom) and levels of eGFP-wt-MRP4 membrane expression were similar to those of cells not treated with monensin (Fig. 5B, top). Quantitative analyses further revealed that monensin induced the localization of eGFP-wt-MRP4 in intracellular vesicles containing LAMP-2 in 98% of cells when treated with control siRNA. In contrast, monensin failed to induce eGFP-wt-MRP4 localization in intracellular vesicles in 75% of cells lacking NHERF1 (P < 0.05; Fig. 5C). Immunoblot analyses of these cells confirmed complete depletion of NHERF1 protein after siRNA treatment, whereas a strong NHERF1 band was observed in control siRNA–treated cells (Fig. 5D, top). In cells where NHERF1 expression was completely abrogated, the levels of MRP4 protein were significantly increased as before (Fig. 5D, middle).

The increase in plasma membrane MRP4 in NHERF1-depleted cells could result from decreased internalization of MRP4 or from increased recycling of the transporter through the endosomes or both. To distinguish among these possibilities, we examined the effects of monensin on MRP4 trafficking because this ionophore selectively inhibits the endocytic recycling of membrane proteins but does not affect their internalization (31, 32). Monensin treatment of NHERF1-depleted cells markedly reduced internalization of eGFP-wt-MRP4 in the majority of cells, and it therefore seems likely that decreased internalization rather than increased recycling is largely responsible for the increased plasma membrane expression of MRP4 that is observed in the absence of NHERF1. Thus, our data support the conclusion that interaction between NHERF1 and MRP4 is required for the internalization of the latter protein.

Similar to MRP4, the internalization of the activation-independent parathyroid hormone receptor PTH1R (which also has a canonical class I PDZ interaction motif) requires NHERF1 (33). In the case of PTH1R, the capacity of NHERF1 to establish cell-specific effects on PTH1R internalization was tested in three independent ways and it was concluded that the role of NHERF1 is not necessarily an “active” one but rather that the interactions between the PTH1R, NHERF1, and the cytoskeleton confer sufficient membrane stability on PTH1R for internalization (33). However, in contrast to our observations here with MRP4 and that reported for PTH1R, it seems that the internalization of wild-type CFTR and its PDZ motif deletion mutant, CFTR-ΔTRL, does not differ in polarized epithelial cells. Instead, recycling of the CFTR-ΔTRL mutant is reduced relative to wild-type CFTR (24). Thus, the role of NHERF1 in MRP4 trafficking to the plasma membrane differs quite dramatically from its role in trafficking of the related CFTR. Intriguingly, it has been reported very recently that MRP4 physically and functionally associates with CFTR via the PDZ scaffolding protein PDZK1 in certain cell types (34). Thus, it seems that inhibition of MRP4-mediated cyclic AMP transport potentiates adenosine-stimulated CFTR chloride currents at apical surfaces of gut epithelia in a microcompartimentalized fashion. Our present studies showing the functional interaction of MRP4 with NHERF1 establishes that the physical association of MRP4 with a second PDZ scaffolding protein can regulate the more global drug efflux activity of this transporter.

Finally, in the case of the β2-adrenergic receptor, expression of this G protein–coupled protein was down-regulated when its interaction with NHERF1 was disrupted, unlike MRP4, which we have shown is up-regulated at the plasma membrane in NHERF1-depleted cells. Thus, in the absence of functional NHERF1, endocytosed β2-adrenergic receptors are sorted to lysosomes (for degradation) instead of being recycled back to the plasma membrane. Therefore, it has been concluded that NHERF1 has a critical role in recycling of β2-adrenergic receptors to the plasma membrane (35). It has also been determined that the serine residue (Ser411) in the PDZ interaction motif of the β2-adrenergic receptor must be phosphorylated by the serine/threonine kinase G protein–coupled receptor kinase-5 in order for proper recycling of the receptor to take place (35). Whether phosphorylation of the threonine residue in the MRP4 PDZ interaction motif ETAL affects its interaction with NHERF1, or internalization of the transporter and, if so, what kinase is involved awaits further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Regulation of MRP4 Expression and Function by NHERF1


Down-regulation of Na\(^+\)/H\(^+\) Exchanger Regulatory Factor 1 Increases Expression and Function of Multidrug Resistance Protein 4

Md. Tozammel Hoque and Susan P.C. Cole


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