Distribution dynamics and functional importance of NHERF1 in regulation of Mrp-2 trafficking in hepatocytes

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MATERIALS AND METHODS

Cell culture. All animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Southern California Institutional Animal Care and Use Committee. WIF-B cells (kindly provided by Dr. Ann L. Hubbard, Johns Hopkins University, Baltimore, MD) were grown at 37°C under 5% CO2 in modified Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

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Coon’s F12 medium supplemented with 5% fetal calf serum and 10 μmol/l hypoxanthine, 0.04 μmol/l aminopterin, and 1.6 μmol/l thy- 
midine, as described elsewhere (21). Cultures were passaged using 
standard trypsinization procedures. Cells were seeded on 3.5-cm 
glass-bottom and 6-cm plastic cell culture dishes at a density of 
~3.8 × 10^6 cells/cm^2. Cells were used when maximal density and 
polarity were reached, typically after 7–10 days of culture. At this 
time, 70–80% of cells formed intercellular phase-lucent, spherical 
structures (apical vacuoles), defined to be bile canalicular-like spaces, 
as described elsewhere (21, 38). Cells were infected with recombinant 
adenoovirus (rAd) at multiplicity of infection of 10 infectious units 
(IU)/cell for 36 h. Hepatocytes were isolated from rat liver by 
collagenase perfusion, as described elsewhere (4, 14, 43). Cells were 
cultured on collagen-coated glass-bottom dishes in Williams E me-
dium with the addition of 5% fetal calf serum, 10 mM HEPES buffer, 
2 mM L-glutamine, 1.8 g/l glucose, 1 μM dexamethasone, 4 mg/l 
insulin, 100 μM penicillin and 100 μg/ml streptomycin, and 10 mg/l 
gentamicin. Cells were overlaid with Matrigel (BD Bioscience, San 
Jose, CA) 24 h after seeding, and the culture was kept under 5% CO_2 
at 37°C for 4 more days before infection. Hepatocytes were infected 
with rAd at multiplicity of infection of 10 IU/cell for 36 h.

**Generation of rAd.** Full-length human NHERF1 gene (gene ID 
9368) was cloned into the shuttle vector pDC311-YFP for generation of 
rAd or into the pGEX-KG vector for a GST pulldown experiment. For 
site-directed mutagenesis, NHERF1 F355R and radixin N210/T214 were 
subcloned into pDC311 vector for generation of the pDC311-
NHEF1 WT and N210/T214 mutant, human GST-NHERF1 full-
ences). The rAd was generated by cotransfection of pDC311 shuttle 
vector pDC311-YFP and pGEX-KG vectors. PCR-

**Membrane-binding assay.** Isolated hepatocytes were infected with 
rAd expressing YFP-NHERF1 WT and YFP-NHERF1 F355R. Un-
infected cells were used as a control. Cells were treated with 1% 
Triton X-100, and cell lysates were centrifuged at 4°C (15,000 g) for 
15 min. YFP-NHERF1-infected and control supernatant and pellet 
fractions were separated by SDS-PAGE. Gels were subjected to 
immunoblotting with anti-NHERF1 antibody. Signals corresponding 
to YFP-NHERF1 WT and YFP-NHERF1 F355R were quantified by 
densitometry, and proportions are reported.

**Immunoblotting and live cell imaging.** Cells were fixed by treat-
ment with 4% formaldehyde and permeabilized with 0.1% Triton 
X-100. Samples were then probed with anti-NHERF1 (kindly 
provided by Dr. Chris Yun, Emory University) and anti-Mrp-2 (Sigma, 
St. Louis, MO) antibodies. Yellow fluorescent protein (YFP) was 
detected using rabbit polyclonal antibody against green fluorescent 
protein (GFP; Immunology Consultants Laboratory, Newberg, OR), which 
also reacts with YFP. For live cell imaging, WIF-B cells and 
hepatocytes were seeded on glass-bottom cell culture dishes and 
infected with rAds. Images were obtained using a fluorescence mi-
roscope (Carl Zeiss Microscopy, Thornwood, NY).

**Glutathione-methylfluorescein secretion by hepatocytes.** Accumu-
lation of glutathione-methylfluorescein (GSMF) into biliary canalic-
ular space was evaluated using the GSMF precursor 5-chloromethyl-
fluorescein diacetate (CMFDA) (2, 31, 44). CMFDA is lipophilic and 
nonfluorescent and freely diffuses into the cell. Cytosolic esterases 
effectively cleave the acetate groups and convert CMFDA to mem-
brane-impermeable fluorescent 5-chloromethylfluorescein, which 
then reacts with intracellular glutathione to produce GSMF. GSMF is 
a substrate of the apical Mrp-2 transporter and is efficiently 
accumulated and accumulated in canaliculalumina with little retention 
in cytosol. For the assay, accumulation of dye by cells in normal medium is compared 
with accumulation of dye by cells in which tight junctions between cells are 
disrupted by Ca^{2+}-free medium, thus liberating the GSMF. Hepatocytes 
under various experimental treatments were first equilibrated in a 
standard buffer consisting of 0.35 g/l KCl, 0.25 g/l MgSO_4, 0.18 g/l 
CaCl_2, 0.16 g/l KHPO_4, 4.8 g/l HEPES, 7.9 g/l NaCl, and 0.9 g/l 
glucose, pH 7.4, at 37°C. Cultures were then preincubated with standard 
buffer or in Ca^{2+}-free standard buffer containing 10 mM EGTA at 37°C 
for 10 min. To start the assay, 2 μM CMFDA was added to the cells with 
and without Ca^{2+}-free medium. After 30 min, cells were washed in PBS and lysed in 1% Triton X-100 in PBS. The cell lysates were measured using a 
FLUostar Omega microplate reader (485-nm excitation, 530-nm emis-
sion; BMG Labtech, Cary, NC) (39, 44). Polarized excretion of 
substrate was quantitatively assessed on the basis of the following 
equation: biliary excretion rate = [(GSMF uptake_{standard buffer} – GSMF uptake_{Ca^{2+}-free buffer})/GSMF uptake_{standard buffer}] × 100, 
where the numerator represents the GSMF uptake measured over 30 
min in treated cells in standard buffer minus that measured for treated 
cells in Ca^{2+}-free buffer and the denominator represents GSMF 
uptake measured in uninfected cells in standard buffer over 30 min.

**Statistical analysis.** Data sets were compared using Student’s 
two-sample t-test. The criterion for significance was at least P < 0.05.

**RESULTS**

**Expression and localization of NHERF1 in hepatocytes.** We 
used several methods to analyze NHERF1 expression in hepa-
tocytes (Fig. 1). Immunoblot analysis showed abundant expres-
sion of NHERF1, as well as Mrp-2 (Fig. 1A). Furthermore, 
NHERF1 and Mrp-2 were colocalized within the canecular plasma 
membrane (Fig. 1B). NHERF1 and Mrp-2 were also identified in pericanalicular membranes but were virtually 
absent in the basolateral compartment.

**YFP-NHERF1 WT and the NHERF1 ΔPDZ2 mutant localize on the 
apical membrane.** Adenovirus containing radixin-CFP WT, YFP-NHERF1 WT, and YFP-NHERF1 ΔPDZ2 was in-
trouced into isolated hepatocytes. Both WT radixin and 
NHERF1, as well as the NHERF1 ΔPDZ2 mutant [i.e., both cyan 
fluorescent protein (CFP) and YFP], were clearly expressed in 
WIF-B cells (Fig. 2A) and primary hepatocytes (Fig. 2B) 
beginning at ~12 h after infection. High levels of expression 
were also observed by 36 h after infection. YFP signals are
similar in cells infected with WT NHERF1 and cells infected with the NHERF1 ∆PDZ2 mutant. YFP is localized on the canalicular membrane, similar to native NHERF1 expression, with relatively no YFP signal on the basolateral membrane. NHERF1 was localized to apical membrane vacuoles, although there was usually some diffuse cytoplasmic NHERF1 labeling. Radixin-CFP WT is also localized on the canalicular membrane. These data indicate that YFP-NHERF1 WT and the NHERF1 ∆PDZ2 mutant are primarily targeted to the canalicular plasma membrane of primary hepatocytes.

Overexpressed YFP-NHERF1 F355R and YFP-NHERF1 ∆PDZ1 mutants are redistributed to cytoplasmic space. To further determine the distribution dynamics of NHERF1, we examined the radixin binding site mutants YFP-NHERF1 F355R and YFP-NHERF1 PDZ1 (Fig. 3). When introduced by adenovirus, their distribution (Fig. 3) was remarkably different from that of either YFP-NHERF1 WT or YFP-NHERF1 ∆PDZ2 (Fig. 2). At 36 h after infection, there was notable cytoplasmic accumulation of the F355R and ∆PDZ1 mutants. In contrast, radixin was localized on the canalicular membrane, similar to YFP-NHERF1 WT expression.

To test whether the NHERF1 F355R point mutation prevents membrane binding, membrane and cytosolic fractions from YFP-NHERF1 WT and YFP-NHERF1 F355R point mutant adenovirus-infected hepatocytes were analyzed by Western blotting (Fig. 4B). Results from Western blot analysis are consistent with the fluorescence microscopic observations that YFP-NHERF1 WT protein was mostly localized in the membrane fraction (67%), whereas YFP-NHERF1 F355R mutant protein was mostly localized in the cytoplasmic fraction (15%) (P < 0.01).

YFP-NHERF1 F355R and YFP-NHERF1 ∆PDZ1 alter the distribution of Mrp-2. We next tested the colocalization of NHERF1 mutants with Mrp-2. In collagen sandwich-cultured hepatocytes infected with YFP-NHERF1 WT and mutant constructs (F355R, ∆PDZ1, and ∆PDZ2) (Fig. 4), we found that YFP-NHERF1 WT and Mrp-2 were localized to the canalicular membrane but not to the basolateral membrane. There was a high degree of colocalization of NHERF1 and Mrp-2 on the canalicular membrane. NHERF1 was localized predominantly to the apical plasma membrane vacuoles, but there also appeared to be a diffuse staining in the cytoplasmic compartment. Mutant YFP-NHERF1 F355R and YFP-NHERF1 ∆PDZ1 proteins were expressed prominently on the cytoplasmic space. Endogenous Mrp-2 was also distributed throughout the cytoplasm (Fig. 4). NHERF1 F355R and NHERF1 ∆PDZ1 mutants were not associated with the canalicular membrane.

Ineffective binding of YFP-NHERF1 F355R and YFP-NHERF1 ∆PDZ1 mutants with Mrp-2. Using an in vitro binding assay (30, 32), we next examined the NHERF1-Mrp-2 interaction in hepatocytes. WT NHERF1 and WT radixin bound Mrp-2, while the NHERF1 binding site mutated GST-radixin N210/T214 bound much less effectively (Fig. 5, A–C). These results indicate that NHERF1 is essential for the radixin-Mrp-2 interaction. To further evaluate the NHERF1-Mrp-2 interaction, we also used GST-tagged NHERF1 PDZ1 and PDZ2 recombinant proteins. NHERF1 PDZ1 was ineffective in binding with Mrp-2 (Fig. 5D). In contrast, NHERF1 ∆PDZ2 and NHERF1 WT effectively interacted with Mrp-2 (Fig. 5D). These data indicate that NHERF1 interacts with Mrp-2 via its PDZ1 domain.

To determine the extent of binding, we quantified in vitro binding data (Fig. 5E). The capacity of Mrp-2 to bind to GST-NHERF1 WT and GST-radixin WT was significant (35% and 33% of GST alone, respectively) compared with control (GST-only fraction). In contrast, Mrp-2 binding was significantly decreased in the presence of the GST-radixin N210/T214 mutant (3% of GST-radixin WT, P < 0.01). Further quantification showed that the GST-NHERF1 PDZ2 domain-deleted mutant was still able to bind to Mrp-2 effectively (27% compared with GST-NHERF1 WT). In contrast, the GST-NHERF1 PDZ1 domain-deleted mutant bound to Mrp-2 less effectively (5% compared with GST-NHERF1 WT, P < 0.01).

Canalicular excretion of fluorescence-tagged Mrp-2 substrate. The canalicular membrane localization of NHERF1 implies that it may play a role in canalicular membrane structure and function. Therefore, we used the CMFDA assay to evaluate the excretory function of Mrp-2 in hepatocytes (4, 39, 44). The secretory response of uninfected and control-infected cells was robust (Fig. 6). The CMFDA secretory response in the YFP-NHERF1

![Fig. 1. Na+/H+ exchanger regulatory factor 1 (NHERF1) expression in hepatocytes. A: hepatocytes were isolated, and cell lysates were subjected to immunoblot analysis to detect NHERF1, which was identified, as expected, at 50 kDa (bottom). Immunoblots were stripped and reprobed with an anti-multidrug resistance-associated protein (Mrp-2) antibody (top). B: WIF-B cells (top) and collagen sandwich-cultured hepatocytes (bottom) were immunolabeled with anti-NHERF1 (green) and anti-Mrp-2 (red) antibodies. Arrows, basolateral membrane; *, canalicular membrane. Representative images, including overlay (merged images), are shown.]
F355R and YFP-NHERF1 PDZ1 domain-deleted mutant-infected cells was decreased significantly compared with control (55% and 45% of control adenovirus, respectively, \( P < 0.01 \)). In contrast, GSMF excretion was significantly increased in YFP-NHERF1 WT-infected cells (128% of control virus-infected cells, \( P < 0.05 \)). GSMF excretion was also significantly reduced in NHERF1 shRNA-infected cells (19% of scramble-infected cells, \( P < 0.01 \); Fig. 6A). These data indicate that NHERF1 is important in canalicular excretory function.

Effect of NHERF1 knockdown on Mrp-2 expression. NHERF1 expression was knocked down in hepatocytes using adenovirus-mediated shRNA (Fig. 6B). To determine if NHERF1 plays a role in the expression of Mrp-2, we analyzed Mrp-2 expression from NHERF1 shRNA-infected hepatocytes. NHERF1 shRNA-infected hepatocytes were analyzed with anti-Mrp-2 antibody, and the expression of Mrp-2 protein was \( \sim 50\% \) of that in scramble shRNA-infected cells (\( P < 0.005 \); Fig. 6C). Our data were consistent with data from a previously published NHERF1-knockout mouse study (30).

**DISCUSSION**

We have examined the effects of deletion of PDZ1 and PDZ2 domains of NHERF1 and radixin binding site mutation of NHERF1 F355R on the targeting and functional participation of Mrp-2 trafficking and function in hepatocytes. The major finding in this study is that Mrp-2 interacts with NHERF1 via its PDZ1 domain and that the radixin binding site and PDZ1 domain of NHERF1 are important for Mrp-2 targeting, distribution, and function. In addition, NHERF1 and Mrp-2 colocalization at the canalicular membrane appears to be functionally critical.

NHERF1 has been found to associate with a number of other transporters and receptors, as well as with a variety of cyto-
plasmic signaling proteins (32, 33). The primary cellular function of NHERF1 is to act as a scaffold protein linking transmembrane proteins, such as transporters, to cytoskeletal proteins, such as ERM proteins. NHERF1, a protein linking radixin and the plasma membrane, is a known component of the hepatocellular microvilli (30). It has been reported previously that three isoforms of NHERF proteins [NHERF1 (EBP-50), NHERF2 (E3KARP), and NHERF3 (PDZK-1)] are expressed in the liver. The last member of the NHERF family, NHERF4 (IKEPP), has not been detected in the liver (6).

We showed specifically that NHERF1 F355R and PDZ1 domain-deleted mutants were incapable of a normal secretory response, while cells overexpressing NHERF1 exhibited an enhanced secretory capability. On the basis of ERM protein (moesin and ezrin) and NHERF1 structural studies, the COOH-terminal 11 residues of NHERF1 appear to bind to the NH$_2$-terminal portion of moesin (11). Further sequence analyses and biochemical studies with different NH$_2$-terminal NHERF1 mutations suggest that the phenylalanine F355 mutant (F355R) abolished interaction with the ezrin NH$_2$-terminal domain (5, 11). We have created a NHERF1 F355R mutation that alters key residues necessary for interaction with radixin on the basis of known moesin structures and sequence comparison. We have already tested our NHERF1 F355R mutant, and we show that F355R does not bind effectively to radixin. Further studies show that F355R mutants have a reduced membrane association compared with WT NHERF1. Thus, overexpression of the F355R point mutant and NHERF-PDZ1 deletion mutant leads
Fig. 4. A: intracellular distribution of YFP-NHERF1 WT and YFP-NHERF1 mutants in collagen sandwich-cultured hepatocytes. Cells were infected with YFP-NHERF1 WT and YFP-NHERF1 mutants (F355R, ΔPDZ1, and ΔPDZ2) and stained for YFP (green) and Mrp-2 (red). Representative images, including overlay, are shown. Arrow, basolateral membrane; *, canalicular membrane. Scale bar = 10 μm. B: membrane localization of YFP-NHERF1 WT and YFP-NHERF1 F355R mutant in hepatocytes. Hepatocytes were infected with recombinant adenovirus expressing YFP-NHERF1 WT and YFP-NHERF1 F355R. After 36 h, membrane and cytosolic fractions were separated. Top: equal fractions of each extraction from all samples were separated by SDS-PAGE and analyzed by immunoblot analysis with anti-NHERF1 antibody. Bottom: data from 3 independent experiments were scanned and quantitated and are presented graphically (proteins from NHERF1 WT and NHERF1 F355R membrane and cytoplasmic fractions were normalized).
to mistargeting of Mrp-2 and changes the secretory phenotype of the hepatocytes. Continued maintenance of functional activity and expression of the YFP-NHERF1 PDZ2 deletion mutant at the apical surface indicate that the PDZ2 domain of NHERF1 is not essential for the interaction between radixin and the plasma membrane or, more likely, that the PDZ1 domain of NHERF1 is sufficient for the morphological and functional phenotype of hepatocytes.

Hegedus et al. (16) showed that the NHERF1 PDZ1 and PDZ2 domains interact with Mrp-2. However, our current data do not support an interaction between Mrp-2 and the NHERF1 PDZ2 domain. Hegedus et al. used small peptides to demonstrate the NHERF1-Mrp-2 interaction. The binding affinity of small peptides is different from that of the PDZ domain deletion mutant of NHERF1 protein. It is possible that nonspecific binding of peptide to Mrp-2 may occur. In addition, clustering peptides may lead to nonspecific binding. Rather, we used PDZ1 and PDZ2 deletion mutants of NHERF1, and our binding assay technique represents a more physiological condition than use of small peptides.

The N210F/T214A ezrin mutant has been created on the basis of the structural studies. Biochemical analyses show that the ezrin (N210/T214) double-mutant NH2-terminal domain failed to bind effectively to NHERF1 compared with WT ezrin (11). To confirm the impact of mutations in the NH2-terminal domain of radixin on NHERF1 binding, we have created an
asparagine 210 and threonine 210 radixin double-mutant
(N210F/T214A). Our binding experiments demonstrated re-
duced binding of Mrp-2 to the radixin double-mutant.

The intramolecular conformation of NHERF1, in which the
COOH-terminal ERM-binding region binds to the PDZ2 do-
main, may be important in its function (5, 11, 32). This
head-to-tail conformation masked the interaction of both
PDZ domains with PDZ domain-specific ligands, such as
phosphatase and tensin homolog (PTEN) and β-catenin. An
ERM binding region composite structure comprising an
α-helix ending in a PDZ binding motif imparted opposite
effects to NHERF1 associations, mediating binding to ERM
proteins and inhibiting binding of PDZ domain ligands. The
PDZ domain inhibition was released by prior association of
ERM proteins with the ERM binding region, a condition that
occurs in vivo and likely disrupts NHERF1 head-to-tail
interaction. The regulation of NHERF1 interactions at the
apical membrane thus appears to be a dynamic process that
is important for maintaining epithelial tissue integrity (32).

Finally, use of NHERF1 also appears to be necessary for

Fig. 6. Canalicular excretion of fluores-
cence-tagged Mrp-2 substrate. A: hepa-
tocytes were treated with fluorescence-tagged
5-chloromethylfluorescein diacetate (CMFDA), and cellular accumulation of CMFDA uptake and excretion were examined 36 h postinfec-
tion with YFP-NHERF1-WT and YFP-
NHERF1 mutants, as well as shRNA constructs.
Biliary excretion index was measured as
percentage of glutathione-methylfluorescein
(GSMF) fluorescence released into canalic-
ular lumina. Data were collected from assays
in 5 separate culture preparations and nor-
malized within each experimental run by set-
ing CMFDA uptake for control (unin-
fected cells) at 100%. *p < 0.05, NHERF1
WT-infected cells vs. control adenovirus
(contains YFP only). ***p < 0.01, NHERF1
F355R and NHERF1 ΔPDZ1 vs. control
adenovirus and NHERF1 shRNA-infected
cells vs. scramble-infected cells. B: hepa-
tocytes were infected with adenoviral con-
structs containing scrambled shRNA (con-
tral) or NHERF1 shRNA for 3 days. Unin-
fected cells were exposed to PBS and served
as an additional control. Cells were har-
vested 1–3 days after infection, and cell
lysates were separated by SDS-PAGE and
subjected to immunoblotting to detect
NHERF1 and F-actin. A representative im-
munoblot is shown. C: data from 3 indepen-
dent experiments were scanned and quanti-
tated and are presented graphically (densito-
metric units were normalized to the band
corresponding to GST alone).
microvillar assembly and function in human choriocarcinoma JEG-3 cells (15).

Our work extends a previous investigation in which the interaction of recombinant Mrp-2 with NHERF1 was originally described (16, 30). We have detailed the role of NHERF1 PDZ domains and the radixin binding site in the localization and functional regulation of Mrp-2 in hepatocytes. Furthermore, NHERF1-knockout mice show a downregulation of Mrp-2 expression from the canalicular membrane, and bile flow and glutathione excretion were significantly reduced (24, 30). Furthermore, while Mrp-2 expression was reduced in NHERF1-deficient mice, Mrp-2 was still localized at the canalicular membrane of hepatocytes (30). This suggests that the Mrp-2-NHERF1 interaction is not required for the apical localization of Mrp-2 or that a functional compensatory mechanism involving other adaptor proteins maintains the apical localization of Mrp-2 in NHERF1-deficient mice. Apical plasma membranes contain a network of scaffolding proteins, such as F-actin, radixin, NHERF1, NHERF2, and PDZK1. These adaptor proteins may compensate for the loss of NHERF1 in NHERF1-deficient hepatocytes in vivo. Thus we speculate that different scaffolding proteins may provide functional redundancy in the apical scaffolding network and can explain the absence of significant phenotypic changes in NHERF1-knockout mice.

NHERF1 has also been reported to interact with and regulate the trafficking of Mrp-4, another ABC transporter that displays dual membrane localization in polarized cells (19, 34, 37). Ectopic expression of NHERF1 in Madin-Darby canine kidney (MDCKI) cells redirects the trafficking of endogenous Mrp-4 from the basolateral to the apical membranes (20). Li et al. (30) found that, unlike Mrp-2, the expression level of hepatic Mrp-4 protein in NHERF1-knockout mice was not significantly changed compared with that in WT mice. This finding is consistent with studies showing that, in hepatocytes, Mrp-4 is localized at the basolateral membrane (34, 37), whereas NHERF1 is expressed at the apical membrane (12, 13).

PDZK1, another member of the NHERF family of scaffolding proteins, has also been reported to interact with human Mrp-2 (25). No change in the distribution or expression level of Mrp-2 was observed in kidney proximal tubule epithelial cells of PDZK1-knockout mice, indicating that the Mrp-2-PDZK1 interaction is not critical for the localization or expression of Mrp-2 in vivo or that functional compensation by other PDZ proteins takes place in PDZK1-knockout mice (26). Similar to our findings, Emi et al. (8) reported that overexpression of the fourth domain of PDZK1 reduced the Mrp-2 localization at the apical membrane, with an increase in intracellular accumulation of mislocalized Mrp-2 in Hep G2 cells.

Little or no mislocalization of radixin was observed in NHERF1 F335R-infected cells, suggesting that the interaction between the NH2 terminus of radixin and NHERF1 is not an absolute requirement for the radixin localization. The COOH terminus of radixin is indiscriminant and has very high affinity for the F-actin binding partner. In addition, we have observed that overexpression of NHERF1 WT was increased by GSMF excretion, suggesting that an excessive amount of NHERF1 was available to transport Mrp-2 to the canalicular membrane.

Our findings indicate that the NHERF1-Mrp-2 interaction might facilitate Mrp-2 recycling from/to the apical membrane.

Fig. 7. Model for NHERF1 interaction with Mrp-2. A: NHERF1 presents a “closed” conformation, in which the PDZ2 domain binds to the COOH-terminal radixin-binding (RB) region in a “head-to-tail” interaction masking the PDZ domains. NH2- and COOH-terminal domains of radixin also bind to each other in a dormant state, preventing COOH-terminal radixin interaction with F-actin and NH2-terminal radixin interaction with NHERF1. B: phosphorylation of the COOH-terminal threonine (T564) induces and stabilizes the unfolded active conformation of radixin, allowing the NH2-terminal domain to bind to the RB region of the NHERF1 and the actin-binding (COOH-terminal) domain to interact with actin filaments. NHERF1 engagement with radixin via the RB region switches NHERF1 to an “open” conformation, in which the PDZ domains are unmasked and PDZ1 is able to bind the COOH terminus of Mrp-2.
under physiological conditions. In the absence of an NHERF1-Mrp-2 interaction, Mrp-2 might accumulate in the cytoplasmic space. It is probable that mutations (F355R and PDZ1 deletion) in NHERF1 might decrease endocytic recycling of Mrp-2 to the apical plasma membrane and, consequently, increase cytoplasmic accumulation of Mrp-2.

A diagram depicting the radixin-NHERF1-Mrp-2 interaction model to promote dynamic bile secretion in hepatocytes is shown in Fig. 7. Both radixin and NHERF1 present a “closed” conformation in hepatocytes. Phosphorylation of the COOH-terminal threonine (T564) by Rho kinase induces and stabilizes the unfolded active conformation of radixin, allowing the NH2-terminal domain to bind to the radixin binding region of NHERF1 and the actin binding domain to interact with actin filaments. NHERF1 engagement with radixin via the radixin binding region switches NHERF1 to an “open” conformation, in which the PDZ domains are unmasked and PDZ1 is able to bind the COOH terminus of Mrp-2. Such a model is logical for cytokoskeleton-Mrp-2 interaction, as well as canalicular Mrp-2 transport and bile secretion. This model also explains why blocking radixin-NHERF1 and NHERF1-Mrp-2 interactions by point mutation or deletions impaired hepatocyte function.

In aggregate, the data strongly suggest that the adaptor protein NHERF1 is essential for the radixin-Mrp-2 interaction and Mrp-2 function. The COOH-terminal cytoplasmic domain of human Mrp-2 has been reported to interact with radixin in vitro, providing for a direct link between Mrp-2 and the actin cytoskeleton (24). Our present findings and previously published data do not support a direct Mrp-2-radixin interaction (30). However, characterization of additional NHERF-regulating proteins, including likely adaptor proteins, protein kinases, and other PDZ proteins, will enhance understanding of the cellular dynamics and function of canalicular transporters.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.K., J.S., and D.C.R. are responsible for conception and design of the research; S.K., J.S., and L.Z. performed the experiments; S.K., J.S., and D.C.R. analyzed the data; S.K. and J.S. interpreted the results of the experiments; S.K., J.S., and D.C.R. prepared the figures; S.K. and J.S. drafted the manuscript; analyzed the data; S.K., and L.Z. performed the experiments; S.K., J.S., and D.C.R. helped support the work reported in this study.

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