Alterations in the proteome of the NHERF2 knockout mouse jejunal brush border membrane vesicles


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Donowitz M, Singh S, Singh P, Chakraborty M, Chen Y, Murtazina R, Gucek M, Cole RN, Zachos NC, Salahuddin FF, Kovbasnjuk O, Broere N, Smalley-Freed WG, Reynolds AB, Hubbard AL, Seidler U, Weinman E, de Jonge HR, Hogema BM, Li X. Alterations in the proteome of the NHERF2 knockout mouse jejunal brush border membrane vesicles. *Physiol Genomics* 43: 674–684, 2011. First published March 22, 2011; doi:10.1152/physiolgenomics.00258.2010.—To identify additional potential functions for the multi-PDZ domain containing protein Na+/H+ exchanger regulatory factor 2 (NHERF2), which is present in the apical domain of intestinal epithelial cells, proteomic studies of mouse jejunal villus epithelial cell brush border membrane vesicles compared wild-type to homozygous NHERF2 knockout FVB mice by a two-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS)-iTRAQ approach. Jejunal architecture appeared normal in NHERF2 null in terms of villus length and crypt depth, Paneth cell number, and microvillus structure by electron microscopy. There was also no change in proliferative activity based on BrdU labeling. Four brush border membrane vesicles (BBMV) preparations from wild-type mouse jejunum were compared with four preparations from NHERF2 knockout mice. LC-MS/MS identified 450 proteins in both matched wild-type and NHERF2 null BBMV; 13 proteins were changed in two or more separate BBMV preparations (9 increased and 4 decreased in NHERF2 null mice), while an additional 92 proteins were changed in a single BBMV preparation (68 increased and 24 decreased in NHERF2 null mice). These proteins were categorized as 1) transport proteins (one increased and two decreased in NHERF2 null); 2) signaling molecules (2 increased in NHERF2 null); 3) cytoskeleton/junctional proteins (4 upregulated and 1 downregulated in NHERF2 null); and 4) metabolic proteins/intrinsic BB proteins (2 upregulated and 1 downregulated in NHERF2 null). Immunoblotting of BBMV was used to validate or extend the findings, demonstrating increase in BBMV of NHERF2 null of MCT1, coronin 3, and ezrin. The proteome of the NHERF2 null mouse small intestinal BB demonstrates up- and downregulation of multiple transport proteins, signaling molecules, cytoskeletal proteins, tight junctional and adherens junction proteins, and proteins involved in metabolism, suggesting involvement of NHERF2 in multiple apical regulatory processes and interactions with luminal contents.

Rationale: NHERF2 is the human homolog of exchanger regulatory factor 2 (ERF2), which has been implicated in the regulation of apical sodium transport in enterocytes. NHERF2 null mice have been shown to have decreased brush border membrane vesicles (BBMV) numbers, which could be due to changes in proliferation, cell size, or microvillus structure. To determine if NHERF2 has a role in these processes, we have performed a proteomic analysis comparing wild-type (WT) with homozygous NH-
(Santa Cruz); alkaline phosphatase (Abcam); Golgin 84 [gift from A. Hubbard, Johns Hopkins University School of Medicine (JHUSOM)]; mitochondrial transporter (gift from P. Pedersen, JHUSOM); coronin 3 [gift from A. A. Noegel, Institute for Biochemistry, University of Cologne (15)].

Cell Isolation and BBMV Preparation

Male WT and NHERF2 homozygous null FVB mice between 12 and 32 wk old were maintained with food/water and prepared for jejunal BBMV proteomic studies as described previously (8, 9) using protocols approved by the Erasmus Medical Center/Dutch Animal Welfare Committee and JHUSOM Animal Care Committee. The breeding and genotyping of all mice studied have been described in detail previously (21a, 30). The jejunal segments were isolated immediately after the animals were killed by overdose of intraperitoneal ketamine plus xylazine followed by cervical dislocation (8, 9). Jejunal segments started just distal to the ligament of Treitz and consisted of ∼50% of the small intestine. Primarily villus cells were obtained by vibration, as described, and then double Mg precipitation was used to isolate BBMV (3, 21a). Four separate preparations of BBMV from age-matched WT and homozygous NHERF2 null animals were studied with both WT and NHERF2 null animals being studied simultaneously. One of these BBMV preparations was made from WT and NHERF2 null mice of pooled jejunal cells from three mice each, and the other three comparisons were BBMV from a single WT mouse paired with a single NHERF2 null mouse. In these studies each BBMV preparation was considered an n = 1.

Characterization of BBMV by Enrichment of Marker Enzymes by Immunoblotting

As described (8, 9), BBMV purification was assessed by immunoblotting (IB) and comparing the amount of proteins in BBMV with that of total membrane preparations, measuring proteins known to be present in brush border (BB) compared with marker enzymes known to reside in specific subcellular organelles. Initially, the total membranes and BBMV had protein concentrations estimated by bichromatic acid assay (BioRad). To further match the amount of protein present, 20 μg of BBMV were separated on one-dimensional SDS-PAGE gels (12%), and the total density of summed proteins was estimated by Ponceau S staining, as described (8, 9). We then separated 20 μg of total membrane and BBMV on 12% SDS-PAGE and compared that for marker enzyme density via IB. The proteins compared localized to: BB, intestinal brush border (BB) and NHERF2 homologous null mice of pooled jejunal cells from three mice each, and the other three comparisons were BBMV from a single WT mouse paired with a single NHERF2 null mouse. In these studies each BBMV preparation was considered an n = 1.

Preparation of BBMV Fractions for Liquid Chromatography-Tandem Mass Spectrometry Including Trypsin Digestion, iTRAQ Labeling, and Liquid Chromatography (Strong Cation Exchange) - Tandem Mass Spectrometry

We TCA-precipitated 150 μg BBMV samples from WT and NHERF2 null animals at a concentration of 1 μg/μl protein, and the pellet was solubilized in TEAB (triethylammonium bicarbonate). Peptides from each sample were differentially labeled using iTRAQ 4-plex (initial pooled BBMV samples) and 8-plex reagents (Applied Biosystems, cat. no. 4390812) according to the manufacturer’s instructions. In brief, 100 μg of protein was dissolved in 1 ml containing 0.5 M TEAB, 0.2% reducing agent [Tris(2-carboxyethyl) phosphine], and 0.1% SDS at 60°C for 1 h; followed by addition of 0.1% cysteine blocking reagent, methyl methanethiosulfonate (MMTS), and kept for 10 min at room temperature. pH of the samples was maintained between 7.5 and 8.0 by adding 0.5 M TEAB. Protein samples were digested using sequencing grade trypsin (1:20 wt/wt, Promega) for 16 h at 37°C. Peptides from each sample were then placed in a final volume of 30 μl and labeled with one of the four- or eight-member iTRAQ reagents in 60 μl of isopropanol at room temperature. After four samples were studied simultaneously, the iTRAQ reagents used (indicated by size added by iTRAQ reagents) were 113, 114, 116, 117 Daltons, and when eight samples were studied the reagents also included (115, 118, 119, 121 Daltons) (8, 9). After 2 h, iTRAQ-labeling reactions were terminated by adding 100 μl water to each sample, and then corresponding samples were combined, and organic solvent was evaporated using a SpeedVac. The pH was adjusted to 3.0 using phosphoric acid and then diluted to 4 ml in strong cation exchange (SCX) solvent A (10 mM potassium phosphate buffer, pH 2.85, in 25% acetonitrile). Combined mixtures of iTRAQ-labeled tryptic digests were fractionated using SCX chromatography on a polysulfoethyl A column (PolyLC, Columbia, MD) (300 A, 5 μm, 100 × 2.1 mm) using an Agilent 1100 HPLC system containing a binary pump, UV detector, and a fraction collector. Fractionation of peptides (0.25 ml fraction) was carried out by a linear gradient between solvent A and solvent B (solvent A plus 350 mM KCl, pH 2.85). The fractions were completely dried and reconstituted in 40 μl of 0.2% formic acid and stored at −80°C until liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed.

MS/MS analysis of iTRAQ-labeled peptides was carried out on a quadrupole time-of-flight mass spectrometer (QSTAR/pulsar, Applied Biosystems). Peptide fractions from SCX chromatography were further separated by reversed-phase LC (Eksigent system) interfaced with a mass spectrometer. RP-LC system consisted of a desalting column (75 μm × 3 cm, C18 material 5–10 μm, 120 A) and an analytical column (75 μm × 10 cm, C18 material 5 μm, 120 A) with a nanoflow solvent delivery at 300 nl/min. Electrospray source was fitted with an emitter tip 8 μm (New Objective, Woburn, MA) and maintained at 2.4 kV electrospray voltage. Peptide samples (40 μl) were loaded onto a trap column in 0.1% formic acid, 5% acetonitrile for 15 min. The gradient was 60 min long (5–40% acetonitrile) with a flow rate of 300 nl/min. Using Analyst v. 1.1 (Applied Biosystems), we acquired MS/MS data by targeting the three most abundant ions in the scan range of m/z 400 to 1,500 Da, and those ions selected were extracted from MS/MS data. Using the case for nonlabeled peptides, 20% higher collision energy was applied during MS/MS scan of ITRAQ-labeled peptides. In addition, “IDA Extension II” hardware script was applied, which guides the mass spectrometer for selecting the peptides for MS/MS based on the ion count threshold selected by the user. This “script in analyst software” was enabled for selection of low-abundance peptides (nearest to threshold ion count >70 with the count every 2 cycles) for fragmentation. This option was expected to widen the proteome coverage compared with regular data-dependent analysis.

Data Analysis and Relative Quantification of Proteins

Analyst raw data files were uploaded to ProteinPilot software version 2.0.1 (Applied Biosystems) and searched against SwissProt database (Swiss-Prot 20070320.fasta and Swiss-Prot 20071204.fasta). ProteinPilot uses the Paragon algorithm for peptide identification, and Pro Group algorithm subsequently processes the searched results. The LC-MS/MS analyses were combined, and relative abundance of proteins was calculated based on individual peptide ratios. Peptide and protein identification was carried out according to the Molecular and Cellular Proteomics guidelines (21). Search parameters included iTRAQ labeling at NH2 terminus and lysine residues, cysteine modification by MMTS, methionine oxidation, and digestion by trypsin. Isoform-specific identification of proteins was carried out by selecting peptides distinct to each form and which excludes all shared peptides from quantitation. Proteins identified with >95% confidence or Proscore >1.3 were used for further analysis.

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Protein identification was based on ProtScore unused score criteria (Pro Group Algorithm, ProteinPilot software). The “unused” ProtScore is a measurement of all the peptide evidence for a protein that is not better explained by a higher ranking protein. It is the true indicator of protein confidence. Identification and quantification of a protein are reported for unique peptides with “unused” confidence threshold (ProtScore) >1.3%.

Bioinformatic Analysis and Categorization of Identified Proteins

After the initial identification of proteins, the list of proteins was searched in the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics, using the proteins’ unique accession numbers, for structure, function, distribution, and subcellular localization. Proteins that appeared to be inadequately described in ExPASy were further searched in Sanger Institute’s collection of protein families and databases (Pfam: http://www.sanger.ac.uk/Software/Pfam/), European Bioinformatics Institute’s database (InterPro: http://www.ebi.ac.uk/interpro/), and the National Library of Medicine and National Institutes of Health’s Entrez PubMed (http://www.ncbi.nlm.nih.gov/pubmed/).

A protein was considered upregulated in the NHERF2 null jejunal BBMV if the ratio of that protein in NHERF2 null/WT was ≥ 1.20 and downregulated if the ratio of that protein in NHERF2 null/WT was ≤ 0.80.

Validation and Extension of Changes of Several BBMV Proteins Identified by the Proteomic Analysis by IB and/or Immunofluorescence Microscopy

Validation of changes in specific proteins was generally dependent on use of IB with occasional use of light microscopy of jejunal segments studied with immunofluorescence and was limited by availability of antibodies to the BBMV proteins identified by MS. In addition, Western blotting was performed of several proteins involved in NHE3 signaling and/or previously shown to be changed in NHERF2 knockout mice by a previous proteomic analysis (9).

Immunohistochemistry. For jejunal histological studies, jejunal sections from WT and NHERF2 null mice were cut open along the mesenteric surface and fixed in 10% neutral-buffered formalin over-night at 4°C. Fixed tissue was embedded vertically in paraffin, and 4 μm sections were prepared, deparaffinized in xylene, rehydrated through a series of graded ethanol exposures, and then stained with hematoxylin and eosin (H&E) or with periodic acid-Schiff (goblet cells). Length of villi and depth of crypts were computed from H&E digital images taken at ×63 on a Zeiss Axios Vert Omer Microscope using MetaMorph software (Roper Industries, Marlton, UK). Numbers of Paneth and goblet cells were counted manually from H&E and periodic acid-Schiff stained slides, respectively, after conversion into digital images, using MetaMorph software. For morphometry, at least 10–15 fields of villi and crypts from each of six mice of each genotype were examined.

Immunofluorescence of Jejunal Segments for β-catenin and p120 was as described (9, 10).

Electron Microscopy of Jejunal BB was as described (9, 10, 21a).

Cell proliferation. We injected 8 to 9 wk old WT and homozygous NHERF2 null mice with bromodeoxyuridine (BrdU, 10 mg/kg ip, Sigma) 2 h before tissue collection (8, 9). After the death of the animals, tissues were fixed in paraformaldehyde (4%) for 4 h at room temperature and embedded in paraffin, and 4 μm sections were cut and applied to Probe On Plus slides, dewaxed and rehydrated through a series of graded alcohols, washed in PBS, and incubated with H2O2 (0.3%) and NaN3 (1 mM) in PBS for 20 min at room temperature. DNA was denatured by incubation of tissues with HCl (1 N) for 15 min at 37°C. Antigen masking was reduced by trypsin digestion (Sigma tablets) for 15 min at 37°C. After being washed in cold PBS, tissues were exposed to Blocking Reagent (Roche) for 1 h at room temperature. Anti-BrdU monoclonal antibody (Sigma) (1:100) was incubated overnight at 4°C (17), and then secondary fluorescent (Alexa Fluor 488) antibodies were exposed for 1 h. Fluorescent image acquisition of tissue was performed with a Zeiss 510 LSM/META confocal imaging system. Eight-bit images were collected and stored. BrdU-labeled cells in crypts were manually counted in a blinded fashion.

RESULTS

Effect of NHERF2 Knockout on Mouse Jejunal Structure, Cell Proliferation, and BBMV Purification

Light microscopic evaluation of jejunal mucosa compared WT and NHERF2 null histology in C57BL/6 mice. Electron microscopy (EM) compared WT and NHERF2 null jejunal microvilli in C57BL/6 mice compared with EM from previously reported FVB WT and NHERF2 null mice (3). The jejunum of WT and NHERF2 null were very similar with no differences in villus length and crypt depth. There was an increase in the number of goblet cells in the villi of the NHERF2 null jejunum and a decrease in the crypt goblet cells but no change in number of Paneth cells (Fig. 1, A–E). In addition, EM of the microvilli revealed no differences between WT and NHERF2 null in microvillus length or number (Fig. 1F). Separately, BrdU labeling at 2 h after intravenous injection was similar in WT and NHERF2 (Fig. 1G), indicating no differences in proliferation rates, unlike the decrease in proliferation seen in NHERF1 null jejunum (9).

There was similar BBMV purification from WT and NHERF2 null mice, with marked and comparable enrichment (measured by IB) of intestinal alkaline phosphatase and reduced concentration of the basolateral marker Na-K-ATPase, while there was minimal contamination with the endoplasmic reticulum (ER) marker calnexin, Golgi marker Golgi 84, and mitochondrial marker phosphate carrier [data not shown but presented for WT BBMV in previous publications (8, 9)].
Thus, we conclude that 1) Changes in the jejunal BBMV of NHERF2 null proteome are not due to structural changes in jejunal villus cells or differences in enrichment of the BBMV or contamination with BLM, ER, Golgi, or mitochondria, and 2) The BBMV preparations were derived predominately from the apical domain with minimal contamination by other jejunal membranes other than BLM, which is de-enriched (Na-K-ATPase: WT BBMV/BBMV actin/WT total membrane/total membrane actin: 0.54 ± 0.09, n = 3; NHERF2 null BBMV/BBMV actin/NHERF2 null total membrane/total membrane actin: 0.57 ± 0.21, n = 3; ns).
Effect of NHERF2 Knockout on Murine Jejunal BBMV Proteome

We identified 450 proteins in both the matched jejunal BBMV from WT mice and the NHERF2 null mouse. This is less than the previously reported 570 proteins identified in WT mouse jejunal BBMV by the same techniques and 463 proteins identified in a comparison of WT and NHERF1 null jejunal BBMV, again using the criterion that a protein must be identified in both WT and NHERF1 null to be considered for comparison of expression levels (8, 9). These lesser numbers of proteins identified in the NHERF2 and NHERF1 studies compared with the reported WT BBMV were due to the requirement we imposed that all proteins listed had to be identified in both WT and NHERF1/2 null of the simultaneously studied BBMV preparations. While using this criterion might miss the largest increases or decreases of proteins in the NHERF1/2 knockout compared with WT BBMV proteome, we based this criterion on the fact that often a given protein was not identified in all BBMV preparations from either WT or NHERF null jejunum.

Changes (increase or decrease) in abundance of 13 proteins were identified in at least two of four matched BBMV preparations from WT and NHERF2 null. Note these comparisons were of single BBMV preparations for WT and NHERF2 null mice except for one preparation in which jejunal villus cells of three WT and three NHERF2 null were pooled (considered a \( n = 1 \)). These 13 proteins were characterized as being 1) transport proteins (one increased, two decreased in NHERF2 null), 2) signaling molecules (2 increased), 3) cytoskeleton proteins (4 increased, 1 decreased), and 4) metabolic proteins and intrinsic BB proteins (2 increased, 1 decreased). Table 1 lists these proteins and includes the number of BBMV preparations in which the changes were found. A similar number of differentially expressed proteins was identified in the pooled preparations (12) and single BBMV preparations (10, 11, 13).

Multiple additional proteins were changed in a single BBMV preparation from NHERF2 null compared with WT mice. A full listing of the classification of proteins identified as changed in NHERF2 null jejunal BBMV is included as Sup-

Table 1. Proteomic studies: alteration in proteins in jejunal BBMV from NHERF2 knockout vs. WT mice presence in two or more BBMV preparations

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of Preparations in Which Protein was</th>
<th>Ratio NHERF2/WT (iTRAQ) (Average)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transport proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocarboxylate transporter 1 (MCT1) (solute carrier family 16 member 1) (SLC16A1)</td>
<td>Upregulated 3/3</td>
<td>1.3</td>
<td>( \text{H}^+ )-coupled anion transporter</td>
</tr>
<tr>
<td>Hephaestin precursor</td>
<td>Downregulated 2/4</td>
<td>0.8</td>
<td>Transcellular iron transport; oxidizes ( \text{Fe}^{2+} ) to ( \text{Fe}^{3+} ) state to allow transferrin binding and to move iron across BLM</td>
</tr>
<tr>
<td>Transmembrane Protein 16F (TMEM16F)</td>
<td>Upregulated 3/4</td>
<td>0.8</td>
<td>( \text{Ca}^{2+} )-activated Cl channel (?)</td>
</tr>
<tr>
<td><strong>Signaling molecules</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-3-3-( \alpha )</td>
<td>Upregulated 2/3</td>
<td>1.2</td>
<td>( \text{G}2 ) cell cycle arrest; regulates P53 half-life; restrict TAZ to cytoplasm</td>
</tr>
<tr>
<td>CECAM1</td>
<td>Upregulated 4/4</td>
<td>1.3</td>
<td>Immunoglobulin superfamily of cell adhesion molecules; also carcinoembryonic antigen family, also called biliary glycoprotein; increases cell proliferation by activating Src; receptor for mouse hepatitis virus</td>
</tr>
<tr>
<td><strong>Cytoskeleton proteins/functional proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokeratin 8</td>
<td>Upregulated 3/3</td>
<td>1.3</td>
<td>Intermediate filament type 2 keratin; needed for endo-/exo-cytosis and polarity maintenance</td>
</tr>
<tr>
<td>Coronin1C (also called Coronin 3)</td>
<td>Upregulated 3/4</td>
<td>1.2</td>
<td>F-actin binding protein that binds &amp; inhibits ARP 2/3 complex; positive regulator of cell motility</td>
</tr>
<tr>
<td>Coronin 2A</td>
<td>Downregulated 3/4</td>
<td>1.4</td>
<td>Same as coronin 1C</td>
</tr>
<tr>
<td>Cofilin 1</td>
<td>Upregulated 2/4</td>
<td>1.2</td>
<td>Actin modulating/binding protein which alters F-actin polymerization</td>
</tr>
<tr>
<td>Crumbs protein homolog 3 precursor</td>
<td>Downregulated 2/2</td>
<td>0.6</td>
<td>Epithelial polarity; associated with tight junctions</td>
</tr>
<tr>
<td><strong>Metabolic proteins/intrinsic BB proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGPRRT</td>
<td>Upregulated 2/2</td>
<td>1.2</td>
<td>Purine salvage</td>
</tr>
<tr>
<td>FABP</td>
<td>Upregulated 3/4</td>
<td>1.3</td>
<td>Intracellular metabolism or transport of long chain fatty acids</td>
</tr>
<tr>
<td>Embryonic Alk phosphatase precursor</td>
<td>Downregulated 4/4</td>
<td>0.7</td>
<td>Detoxify LPS</td>
</tr>
</tbody>
</table>

Proteomic identification of each protein is in Supplementary Table S4.
Changes in NHERF2-dependent Jejunal BBMV Proteins by Category

Transport proteins. Several transporters were altered in expression in multiple BBMV preparations from WT and NHERF2 null mice (Table 1, Supplementary Tables S1–S4). These include 1) increased: MCT1 (SLC16A1), a H^+–coupled anion transporter that transports monocarboxylates. It was validated as having increased total membrane expression in NHERF2 null mouse jejenum by IB (Fig. 2B) and was also increased in NHERF1 null jejunal BBMV (9) and 2) decreased: hephaestin precursor [which is involved in oxidizing iron from the Fe^{2+} to Fe^{3+} state and perhaps in movement of iron from the enterocyte apical domain to the BLM domain (37, 52)] and TMEM16F, which is an eight membrane-spanning domain protein that is a member of the recently recognized TMEM 16 family, several members of which include the long-sought epithelial Ca^{2+}–activated Cl channels (31, 38). We identified six peptides of TMEM16F, which provided a very high probability that this protein was correctly identified in jejunal BBMV.

There were multiple additional transport proteins that were altered in expression in a single BBMV preparations when NHERF2 null mice were compared with WT. These are included in Supplementary Table S1. None of the transporters identified in a single BBMV preparation of NHERF2 vs. WT has been validated; however, several of these were changed in expression in BBMV not only comparing NHERF2 to WT but also when comparing BBMV from NHERF1 compared with WT (Supplementary Table S3). Of these, in addition to MCT1, which was increased in multiple BBMV preparations from NHERF2, as well as NHERF1 null mice, the large neutral aa transporter small subunit 2 (SLC7A8) was increased in BBMV from both NHERF1 and NHERF2 null mice (6, 48). All other transport proteins suggested as being altered in NHERF2 null BBMV and also altered in NHERF1 null BBMV were either increased in NHERF1 null and decreased in NHERF2 null or vice versa. For instance, expression of the gene product of downregulated in adenoma, DRA, was upregulated in NHERF2 and downregulated in NHERF1 null BBMV. DRA is an apical membrane Cl/HCO3 exchanger and member of the SLC26A gene family that is linked functionally to the BB Na/H exchanger NHE3 to make up colonic and perhaps some small intestinal neutral NaCl absorption (25).

Signaling molecules. Two signaling molecules were increased in BBMV from NHERF2 null mice compared with WT, CECA1 and 14-3-3-ε. CECA1 (also known as biliary glycoprotein and CD66a) is a member of the immunoglobulin superfamily of cell adhesion molecules and is a member of the

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1 The online version of this article contains supplemental material.
gene family that include carcinoembryonic antigen (CECAM5) (10). It is a receptor for mouse hepatitis virus and increases cell proliferation by activating Src (10). 14-3-3 was previously reported to bind to NHERF2-associating protein TAZ (22). TAZ is a transcription factor that under basal conditions is present in the plasma membrane where it binds to PDZ domain 1 of NHERF2 (22). It uses the same domain to bind an unidentified PDZ protein, which holds it in the nucleus to affect transcriptional coactivation. Upon phosphorylation, TAZ associates with 14-3-3, which inhibits its transcriptional activity by inducing nuclear export into the cytosol. How TAZ functions in transcriptional regulation is only partially understood, especially what determines the relative amount of TAZ in its multiple cellular subdomains at any time. It has been suggested that TAZ links the plasma membrane to regulation of transcription and indicates a potential role for NHERF2 in regulation of transcription (22).

An additional signaling molecule, the leucine-rich repeat-containing protein 1 (also called the LANO adapter protein), which was only upregulated in a single BBMV preparation of NHERF2 null jejunum, was also upregulated in BBMV from NHERF1 null mice compared with WT. Several other proteins were oppositely altered in NHERF1 and NHERF2 null BBMV, including 1) the PDZ and LIM domain protein 5 (also referred to as Enigma-like PDZ and LIM domain protein 5), 2) T complex protein 1 subunit-β (both downregulated in BBMV from NHER2 null and upregulated in NHERF1 null compared with WT), and 3) galectin-4, which was upregulated in BBMV from NHERF2 null and downregulated in NHERF1 null BBMV.

Cytoskeleton/junction proteins. BBMV from NHERF2 null mice had increased expression of several cytoskeleton-associated proteins. These include 1) cytokeratin 8 (also increased in BBMV from NHERF1 null mice), 2) ezrin, 3) coronin 1C (also called coronin 3), 4) coronin 2A, and 5) coflin. Cytokeratin 8 is a type 2 intermediate filament known to be present within the terminal web of small intestinal and colonic epithelial cells (2, 33, 42). It interacts with ezrin, and when knocked out, the resulting intestinal epithelial cells have less and shorter microvilli, have less apical ezrin, and lack apical CFTR, alkaline phosphatase, and sucrase-isomaltase. Cytokeratin 8 thus provides mechanical strength to the apical domain of epithelial cells but also appears involved in establishing epithelial polarity and targeting as well as trafficking. Of note, given the lack of structural changes in BB from NHERF2 null mice, overexpression of cytokeratin 8 appears not to alter BB. Coronin 1C (also called coronin 3) and coronin 2A were increased in NHERF2 null jejunal BBMV. Coronins are F-actin-binding proteins and directly bind to and inhibit the Arp2/3 complex, although their role in epithelial cells is not understood (19, 34, 45). The upregulation of coronin 1C in the NHERF2 null mouse jejunum was validated by Western blotting of BBMV (Fig. 2). Cofilin binds and depolymerizes F-actin and inhibits polymerization of G-actin (35). It is phosphorylated and inactivated by LIM kinases and has been shown to regulate apical membrane albumin uptake in renal proximal tubule cells by a mechanism involving CIC-5, which is involved in apical membrane regulated trafficking (18).

In addition, BBMV from NHERF2 null mice had decreased expression of Crumbs protein homology 3 precursor. Crumbs protein homolog 3 is localized at tight junctions and is involved in formation and maintenance of tight junctions and epithelial cell polarity. Myosin XV was decreased in a single NHERF2 null BBMV preparation. It is a nonconventional myosin that has not previously been reported to be present in intestinal epithelial cells.

Proteins involved in metabolism. In considering the significance of changes in proteins involved in cell metabolism, we think it is important to consider that these are usually cytosolic or intracellular organellar proteins, and thus we attempted to de-enrich for their presence. Thus, as “contaminants” of our BBMV preparation, their inclusion is likely to be less reliable quantitatively than proteins that are intrinsic to the apical domain. For that reason, these are listed in Table 1 and Supplementary Tables S1 and S2 but not otherwise mentioned. The only exception is a GPI-anchored protein present on the outer leaflet of the BB, embryonic alkaline phosphatase inhibitor, which was downregulated in BBMV from NHERF2 null mouse jejunum and upregulated in BBMV from NHERF1 null mouse jejunum.

β-Catenin, E-cadherin, p120. Results with β-catenin, E-cadherin, and p120 were more complex. The amount of expression of β-catenin, E-cadherin, and p120 in total membrane from jejunal villus cells of NHERF2 null mice was reduced compared with WT, although not to the same extent as seen with comparably enriched total membrane from NHERF1 null mice prepared at the same time (Fig. 3A, left). This was confirmed by immunofluorescence for β-catenin and p120 (results were less clear for E-cadherin) (Fig. 4). In contrast, proteomic analysis showed no change in the BBMV amounts of these three proteins, while Western blotting demonstrated increased amounts of BBMV β-catenin, E-cadherin, and p120 (Fig. 3A, right). Similar apparently contradictory results were also found in studies of the jejunal BBMV proteome of NHERF1 null compared with WT mice. Similar to the study of NHERF1 null BBMV (9), saponin treating BBMV from WT and NHERF2 null mouse jejunum demonstrated there was less postaspin B-catenin in NHERF2 null compared with WT membranes (Fig. 3B). As we previously described (9), these results show that there are reduced amounts of β-catenin, E-cadherin, and p120 in BB from NHERF2 null compared with WT mice, as also occurred with NHERF1 null (9). We speculate that the increased amount of BBMV protein determined by IB is due to increased cytosolic trapping or loose association of proteins with the preparation, although why these would be present in increased amounts in NHERF2 and NHERF1 null BBMV is unclear (9).

DISCUSSION

This proteomic study was performed to provide insights into functions of NHERF2 in the small intestine on the assumption that changes in protein expression due to absence of NHERF2 might point to previously unidentified direct or indirect functions of NHERF2. We hypothesized that changes would occur even in the likely presence of some compensation expected to occur via contributions of the three other members of the NHERF gene family, which like NHERF2 are present in the apical domain of small intestinal absorptive cells. This study concentrated on the apical domain of mouse jejunal villus absorptive cells since this is the primary localization of NHERF2 although other pools of NHERF2 have been reported for specific
cell types, including juxtanuclear and nuclear localization (22, 45). Expression of the following classes of proteins were altered in NHERF2 null mice: transport proteins, signaling molecules, cytoskeleton components, junctional proteins, and in addition proteins involved in cell metabolism, intrinsic BB proteins, E-cadherin-β-catenin/p120-related proteins, and a small number of proteins without known function. The classification of the proteins altered is not surprising given the multiple previously identified functions of NHERF2 and the fact that multiple NHERF2 did not affect proliferation in mouse jejunum as judged by BrdU staining. The latter was supported by an siRNA study in HeLa cells performed by

![Fig. 3](image_url)

**Fig. 3. A**: Western blots from total membranes (left) and BBMV (right) showing increased BBMV but decreased total β-catenin, E-cadherin, and p120 in NHERF2 null jejunum. Total membranes and BBMV were made from WT and NHERF2 null mouse jejunum. SDS-PAGE and Western blots were performed for each protein. Odyssey Licor quantitation of the BBMV amounts of protein normalized to β-actin was determined, and results are expressed as means ± SE with P values comparing WT and NHERF2 null. **B**: saponin-treated/washed BBMV have reduced β-catenin. BBMV were saponin-treated and washed, and Western blots were analyzed for β-catenin expression, as described (39). Similar results were found in 2 experiments.

![Fig. 4](image_url)

**Fig. 4**. p120 and β-catenin are reduced in jejunal villus epithelial cells of NHERF2 null mice. Light microscopic sections of jejunum were prepared from WT and NHERF2 null mice and stained with anti-p120 and β-catenin antibodies. Bar is 60 μm. Similar results were seen in 2 separate experiments from 4 mice.
Merck in which NHERF2 kd was not associated with a change in proliferation (results kindly provided by C. Buser-Doepner and S. H. Friend with permission of Merck).

NHERF2 previously has been shown to have the following functions: I) scaffolding for establishing subcellular localization of multiple proteins. This includes effects on transport proteins, including CFTR, GPCR (PTH R1) and receptor tyrosine kinases (PDGFR); and other proteins such as the glomerular podocalyxin (12, 20, 21a, 29, 39, 41). 2) Trafficking of proteins to specific subcellular domains: identified examples include effects on the K channel Kv15 (voltage gated); Ca\(^{2+}\)-dependent endocytosis and LPA-stimulated exocytosis of NHE3; albumin endocytosis, which appears to require NHERF2 via a process involving CIC-5; and MCC (mutated in colorectal cancer) role in cell migration (1, 5, 17, 26–28). 3) Complex formation: examples include NHE3 and its stimulation by LPA (5, 28) and inhibition by cAMP, cGMP, and elevated Ca\(^{2+}\); and PDGF stimulation of proliferation by a PI3-K/Akt-dependent process, which also involves ERK (MEK independent) and Src (6, 30, 48). 4) Enzyme function: Ser/Thr kinase and phospholipase functions, including SGK1 and PLC\(\beta\)3 effects, are greatly increased by NHERF2; TRPV and NHE3 both require NHERF2 to respond to SGK1; and PTH R1 shifts signaling from adenylyl cyclase-cAMP to PLC-Ca\(^{2+}\) when bound to NHERF2 (5, 11, 53).

Related to those functions, NHERF2 has been shown to directly bind many of the above proteins. The proteins shown to physically associate with NHERF2 include I) transport proteins NHE3, CFTR, PEP2T, ROMK, plasma membrane Ca\(^{2+}\)-ATPase; 2) signaling molecules PLC\(\beta\)3, PTEN, PDGFR, Sry1, LPA2R, LPA5R, and SGK1, and 3) others: podocalyxin and MCC (mutated in colorectal cancer). It is tempting to speculate how changes in some of the proteins identified in this study might relate to regulation of specific apical processes in the intestine. For instance, are the changes in coronin 1C, which binds Arp2/3 and inhibits actin nucleation (19, 35, 46), relevant to the actin rearrangement that accompanies regulation of NHE3 by trafficking, or is coronin 1C involved in other aspects of NHE3 trafficking? Of note, the current study has not delved into mechanisms, and findings are only meant to be used to suggest future mechanistic studies.

It was surprising that this proteomic analysis did not identify changes in the expression of any of the small intestinal apical membrane proteins previously shown to bind to NHERF2 (listed above), except a single preparation that identified NHE3, even though many of these proteins are expressed in the apical membrane of the jejunal villus absorptive cells studied. The approach we used for protein identification is based on membrane purification (enrichment of the apical domain) followed by trypsinization to produce protein fragments that are identified by MS. Thus failure to identify a protein is not equivalent to lack of expression of the protein.

Nonetheless, the findings of these studies contribute to understanding of intestinal epithelial cell biology partially by identifying intestinal epithelial localization of proteins and suggesting apical domain subcellular localization of proteins not previously known to even be present in intestinal epithelial cells (such proteins include myosin XV, bassoon, and TMEM 16F). Message for the latter, but not protein expression, had been demonstrated previously in the intestine. TMEM 16F is a member of the recently identified anoctamin gene family, some members of which are Ca\(^{2+}\)-activated Cl channels (31, 37). This is the initial suggestion that TMEM16F at the protein level is expressed in intestine, although message has been identified ubiquitously for TMEM16F, including in all intestinal segments with largest amounts in colon (31). There is some preliminary but nondefinitive evidence that TMEM16F functions as a Cl channel (38). This finding warrants further study as to whether TMEM16F functions as a Ca\(^{2+}\)-activated Cl channel or regulator of these channels in the murine small intestine. Whether TMEM16F interacts with NHERF2 in vivo is also unknown although it lacks a COOH-terminal type 1 PDZ domain binding site (KTE), which is the type of PDZ binding domain with which NHERF2 has been shown to associate.

Insights are provided by these studies in signaling complexes potentially of importance for cell-cell interactions that might involve NHERF2. While our results demonstrate a shift to the plasma membrane (either lateral tags of this BBMV preparation or trapped cytosol) of \(\beta\)-catenin, p120, and E-cadherin, how this relates to the overall reduction of these proteins in the NHERF2 null mouse jejunum has not been resolved. However, given that NHERF2 knock does not affect intestinal proliferation while the WNT pathway is critical for normal intestinal turnover is against these changes relating to an alteration in WNT signaling. These NHERF2 effects on p120, E-cadherin, and \(\beta\)-catenin were similar to the findings in the jejunal BBMV from the NHERF1 knockout mouse, as we reported (94). Kreimann et al. (24) made similar observations, which they attributed to the tumor suppressor function of NHERF1 and the subsequently reduced proliferation of the NHERF1 knockout mouse. Of note is lack of effect on proliferation of NHERF2 and lack of evidence that NHERF2 has tumor suppressor activity, suggesting these changes with NHERF2 knockout do not occur by that proposed mechanism. We were unable to find other examples of cell systems in which changes in signaling were associated with reduced \(\beta\)-catenin, E-cadherin, or p120, while plasma membrane amounts or trapped cytosol were increased. However, these results constitute a warning that proteomic studies of closed molecular components can be confounded by trapping of cytosol or contamination within other membranes.

Other proteins that had validated increased expression in NHERF2 null BBMV were coronin 1C and 2A. The role of these F-actin-binding proteins in epithelial cells is not understood despite their binding and inhibiting the Arp2/3 complex, the function of which is to create branching complexity of the actin cytoskeleton (19, 35, 46). However, recently, a coronin was shown to segregate with a part of the recycling endosome that formed tubules that contained the \(\beta\)-2-adrenergic receptor (32). Coronin, actin, and cortactin colocalized in this domain of the recycling tubular endosome, suggesting this complex has a role in regulated recycling (32), a function NHERF2 appears to share. Whether coronins bind any NHERF and whether or how they function together are not known. NHERF2 null mouse jejunum BBMV has a proteome in which there are changes in transport proteins, signaling molecules, cytoskeleton and junction proteins, and proteins involved in metabolism. The identification of changes in expression of proteins in NHERF2 null mice should lead to studies asking whether NHERF2 has a regulatory role in the function of these proteins, although indirect as well as direct effects could account for the changes.
in protein expression demonstrated. For instance, the role of NHERF2 in regulation of MCT1, hephaestin precursor, or TMEM16F is likely to provide insights not only in their regulation but also in defining their functions, which may not occur in the absence of NHERF2. However, it is important to add that lack of identification of known NHERF2-binding partners, which was shown in the past to be present in the intestinal BB, shows that the approach taken in this study will not provide a comprehensive description of NHERF2-interacting proteins. Furthermore, while these proteomic studies were only carried out in murine small intestine, similar studies in other epithelia such as colon, gall bladder, or proximal tubule have potential to provide insights in differences of regulation affected by NHERF2 among these tissues.

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DISCLOSURES

M. Donowitz is a partial owner of Tranzmembrane a company that holds the NHE3 patent.

REFERENCES


