Sorting nexin 27 regulates basal and stimulated brush border trafficking of NHE3

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ABSTRACT Sorting nexin 27 (SNX27) contains a PDZ domain that is phylogenetically related to the PDZ domains of the NHERF proteins. Studies on nonepithelial cells have shown that this protein is located in endosomes, where it regulates trafficking of cargo proteins in a PDZ domain-dependent manner. However, the role of SNX27 in trafficking of cargo proteins in epithelial cells has not been adequately explored. Here we show that SNX27 directly interacts with NHE3 (C-terminus) primarily through the SNX27 PDZ domain. A combination of knockdown and reconstitution experiments with wild type and a PDZ domain mutant (GYGF → GAGA) of SNX27 demonstrate that the PDZ domain of SNX27 is required to maintain basal NHE3 activity and surface expression of NHE3 in polarized epithelial cells. Biotinylation-based recycling and degradation studies in intestinal epithelial cells show that SNX27 is required for the exocytosis (not endocytosis) of NHE3 from early endosome to plasma membrane. SNX27 is also required to regulate the retention of NHE3 on the plasma membrane. The findings of the present study extend our understanding of PDZ-mediated recycling of cargo proteins from endosome to plasma membrane in epithelial cells.

INTRODUCTION

In polarized epithelial cells, the representation of domain-specific integral membrane proteins, including transporters, involves trafficking machinery between intracellular organelles, and the trans-Golgi, endosomes, and lysosomes. The balance of endocytosis, exocytosis, plasma membrane stability, and sorting to breakdown and synthetic pathways determines plasma membrane residence. Protein–protein interactions are involved in multiple aspects of protein residence in intracellular and plasma membrane compartments, and a common motif involved is the postsynaptic density protein 95/discs large/zona occludens 1 (PDZ) domain. For instance, the single-PDZ-domain–containing protein cystic fibrosis transmembrane conductance regulator (CFTR)–associated ligand (CAL) contributes to setting the balance of CFTR in the synthetic compartment, lysosomes, and plasma membrane (Cheng et al., 2002). The involvement of PDZ domain–containing proteins in setting plasma membrane expression of trafficked proteins has also been demonstrated for G protein–coupled receptors, including the thyrotropin receptor, the somatostatin receptor subtype 5, CFTR, the β2-adrenoreceptor (β2AR), and Na+/H+ exchanger 3 (NHE3; B.C., unpublished data; Swiatecka-Urban et al., 2002; Lahuna et al., 2005; Wente et al., 2005; Lauffer et al., 2010). As another example, the endogenous intestinal and renal brush border Na+/H+ antiporter NHE3, which contributes to the majority of intestinal and renal sodium and water absorption, has a total half-life of ∼16 h, although it cycles between the plasma membrane and endosomal compartment (Cavet et al., 2001; Donowitz et al., 2005; Cha et al., 2006; Singh et al., 2014b). The balance between endocytosis and exocytosis of NHE3 is partially dependent on its binding PDZ domain–containing proteins. The identified brush border PDZ domain–containing proteins that affect NHE3 include the Na+/H+ exchange regulatory cofactor (NHERF) family of multi-PDZ-domain–containing scaffolding proteins (NHERF1–4) and the single-PDZ-domain–containing protein Shank2 (Donowitz et al., 2005; Han et al., 2006). The NHERF family was named because of...
its role in NHE3 regulation, including helping set the balance of NHE3 in the brush border versus the endosomal compartment. The NHERF proteins take part in multiple aspects of the regulated trafficking of NHE3; for instance, NHERF1–3 are involved in cAMP inhibition, whereas NHERF2 is necessary for stimulation of NHE3 by lysophosphatidic acid and c-glucose and inhibition by protein kinase C and cGMP/CytK (Lee-Kwon et al., 2003; Cha et al., 2005, 2010; Cinar et al., 2007; Murtazina et al., 2007, 2011; Zachos et al., 2009; Lin et al., 2011a; Sarker et al., 2011). Nonetheless, how the NHERF proteins regulate the balance of NHE3 between the plasma membrane and endosomes has remained unclear, since the NHERFs are primarily localized to the plasma membrane, with no identified endosomal localization.

Insights into the possible roles that PDZ adapter proteins play in the endosomes come from the phylogenetic analysis of all mammalian proteins, which reveal that the NHERF-family PDZ domain sequences are related to the PDZ domain of sorting nexin 27 (SNX27), the only known PDZ domain–containing protein identified in endosomes (M. Donowitz, A. Sharma, and C. Brett, unpublished results; Joubert et al., 2004). In fact, NHERF-family PDZ domain sequence homology was greater with SNX27 than with any other mammalian protein other than the homology among the four members of the NHERF family.

SNX27 is a member of the sorting nexin family of proteins, which are a diverse group of cytoplasmic and membrane-associated proteins implicated in endocytosis and protein trafficking. This protein family is characterized by the presence of a phosphoinositide-binding PX domain, which targets proteins to phosphatidylinositol-3-monophosphate–rich membranes of the endosomal system (Carlton and Cullen, 2005; Cullen, 2008; van Weering et al., 2010). SNX27 is unique among the PX proteins in containing an N-terminal PDZ domain that is upstream of the PX domain. SNX27 has also been annotated to possess a Ras-association domain and an unusual FERM domain upstream of the PX domain and an unusual band 4.1/ezrin/radixin/moesin (FERM)-like structure (Figure 1B). To determine more about the NHE3-SNX27 interaction, five glutathione S-transferase (GST)–SNX27 fusion proteins were purified: 1) GST-SNX27-FL (full length, amino acids [aa] 1–539); 2) PDZ (aa 1–156); 3) SNX27-F (FERM aa 272–539); 4) PDZ + PX (aa 1–266); and 5) PX + FERM (aa 158–539). These GST proteins were used to pull down NHE3 from cell lysate containing HA-NHE3. GST-SNX27 fusion proteins or GST control protein were incubated with HEK-HA-NHE3 cell lysates. After washing, proteins bound to GST beads were separated by SDS–PAGE and identified by anti-HA (NHE3) or anti-GST (SNX27) antibodies (Figure 1C). Based on the pull-down assays, in addition to SNX27-FL, NHE3 interacts with PDZ and PDZ + PX fusion proteins. It is important to note that further extension of the PDZ domain to the PDZ domain significantly increased the binding of NHE3 with the PDZ domain of SNX27 (Figure 1C). Weak but significant binding was also seen with FERM and PX-FERM domains of SNX27; however, further studies are needed to understand the physiological relevance of this weaker binding.

We also asked whether SNX27–PDZ interaction is via the PDZ motif at the C-terminus of NHE3 (-S-T-H-M). To determine this, we created a HA-NHE3ΔC4 (-S-T-H-M) construct in HEK-293 cells and used GST-SNX27-PDZ-PX fusion proteins or GST control protein to pull down HEK-HA-NHE3 or HEK-HA-NHE3ΔC4 as before. Unlike with HA-NHE3, the binding of SNX27-PDZ-PX was greatly reduced with deletion of the last four amino acids at the C-terminus of NHE3 (Figure 1D).

To confirm direct binding between NHE3 and SNX27, we also performed in vitro pull-down assays using the NHE3 C-terminal (aa 642–832) construct and SNX27-FL fusion proteins (Figure 1E). These results further showed that the interaction between NHE3 and SNX27 is direct. Together these results showed that NHE3 directly interacts with SNX27, which is primarily mediated by the PDZ domain of SNX27.

SNX27 regulates basal NHE3 activity and surface expression

The functional role of the SNX27 binding to NHE3 was next evaluated in SK-CO15 cells, which are derived from a human adenocarcinoma of the colon. These cells form a polarized epithelial monolayer, with formation of microvilli (Yoo et al., 2012). These cells were selected for functional studies because they express both endogenous NHE3 and SNX27. A lentivirus-mediated delivery system was used to stably knock down SNX27 in these cells (Figure 2, A and B). The SNX27 protein expression was reduced by >80% in SNX27 short hairpin RNA (shRNA) lentivirus–infected cells (Figure 2B). Furthermore, this reduction was maintained in culture medium containing puromycin. Quantitative PCR confirmed that the reduced NHE3 activity in SNX27-depleted cells was not caused by transcriptional down-regulation of NHE3 (no change in mRNA; unpublished data). These cells were further used to determine the effect of knockdown on NHE3 basal activity and surface expression. SNX27-knockdown cells had ~55% reduced basal NHE3 activity (Figure 2C); cell surface biotinylation showed that SNX27–KD caused a ~60% reduced NHE3 surface expression (Figure 2, D and E); however, total NHE3 expression was not changed with SNX27 knockdown (Figure 2D). Similar effects of SNX27 knockdown (Figure 2, A and B) were observed in HEK-293-HA-NHE3 cells (Figure 3, C and E). Taken together, these results show that in intestinal epithelial cells, SNX27 is required to maintain normal NHE3 basal activity and cell surface expression. There was no

RESULTS
Identification of NHE3 as an SNX27 binding partner

To expand the understanding of the role played by SNX27 as an adaptor in endocytic trafficking of NHE3, we used an immunoprecipitation (IP) approach to identify whether SNX27 associated with NHE3 under basal conditions. CoIP was performed with lysates prepared from the polarized intestinal Na+ absorptive epithelial cell line Caco-2BBe, which express endogenous SNX27 (Figure 1A). Because the endogenous expression of NHE3 was too low to perform biochemical studies, these cells were infected with adenovirus containing hemagglutinin (HA)-NHE3. As shown in Figure 1A, SNX27 communoprecipitates with NHE3. This binding was further confirmed by in vitro pull-down assays. SNX27 belongs to the PX domain (Phox homology)–containing protein family but has a unique N-terminal PDZ domain upstream of the PX domain and an unusual band 4.1/ezrin/radixin/moesin (FERM)-like structure (Figure 1B). To determine more about the NHE3–SNX27 interaction, five glutathione S-transferase (GST)–SNX27 fusion proteins were purified: 1) GST-SNX27-FL (full length, amino acids [aa] 1–539); 2) PDZ (aa 1–156); 3) SNX27-F (FERM aa 272–539); 4) PDZ + PX (aa 1–266); and 5) PX + FERM (aa 158–539). These GST proteins were used to pull down NHE3 from cell lysate containing HA-NHE3. GST-SNX27 fusion proteins or GST control protein were incubated with HEK-HA-NHE3 cell lysates. After washing, proteins bound to GST beads were separated by SDS–PAGE and identified by anti-HA (NHE3) or anti-GST (SNX27) antibodies (Figure 1C). Based on the pull-down assays, in addition to SNX27-FL, NHE3 interacts with PDZ and PDZ + PX fusion proteins. It is important to note that further extension of the PDZ domain to the PDZ domain significantly increased the binding of NHE3 with the PDZ domain of SNX27 (Figure 1C). Weak but significant binding was also seen with FERM and PX-FERM domains of SNX27; however, further studies are needed to understand the physiological relevance of this weaker binding.

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**FIGURE 1:** SNX27 and NHE3 directly interact with each other. (A) Interaction between endogenous SNX27 and NHE3 in Caco-2-HA-NHE3 cells. NHE3 (HA-NHE3) was immunoprecipitated (IP) from total lysate of Caco-2BBe cells using anti-HA antibody. Immunoprecipitated samples were subjected to Western blot analysis (IB) and probed with HA and SNX27 antibodies. Representative result from three independent experiments with similar results. (B) Schematic of SNX27 PDZ domain organization showing the PDZ domain, the PX domain, and the FERM domain of SNX27. Expanded box shows sequence comparison of the SNX27 PDZ domain with the first PDZ domain of NHERF1 and NHERF2. Conserved residues in the three PDZ domains are shown in bold. (C) HEK-HA-NHE3 cell extracts (1 mg) were incubated with GST or GST-fusion proteins: 1 nmol; SNX27-FL (aa 1–539), PDZ (P; aa 1–156), FERM (F; aa 272–539), PDZ+PX (P+PX; aa 1–266), PX+FERM (PX+F; aa 158–539). The presence of NHE3 in GST pull downs was detected by Western blotting (top). Equal GST loading was verified by Western blotting for GST. Representative results from three independent experiments. (D) HEK-HA-NHE3 or HA-NHE3ΔC4 cell extracts (1 mg) were incubated with GST or GST-fusion proteins (1 nmol; SNX27- PDZ+PX) and then subjected to pull down with GSH resin. The presence of NHE3 in GST pull downs was detected by Western blotting (top). Representative results from three independent experiments. (E) GST or GST-SNX27-FL was mixed with MBP-NHE3-C-term (aa 642–832) and then subjected to pull down with GSH resin. Samples were analyzed by Western blot (IB) with antibodies against MBP and GST. The experiment was repeated three times, and one representative result is shown.

**effect on the expression levels of NHERF1 or ezrin in these knockdown cells (Figure 2A), suggesting that the SNX27-mediated regulation of NHE3 is not mediated by changes in the expression of some of the other major proteins involved in NHE3 regulation. The lack of enhanced degradation for total NHE3 is interesting and specific to NHE3, as the other cargo proteins known to be regulated by SNX27, such as Glut-1 and β2AR, showed less protein expression in SNX27-KD HEK-293 and SK-CO15 cells (Figure 2F).**
PDZ domain of SNX27 maintains plasma membrane localization and activity of NHE3

To examine the specificity of the SNX27-knockdown effect, we investigated the rescue using rat-derived SNX27b constructs not targeted by the human-specific shRNA lentivirus. Selective knockdown (KD) and replacement were done in HEK-293-HA-NHE3 cells (easier to transfet than with SK-CO15 cells). KD was confirmed by anti-SNX27 immunoblotting (Figure 3, A and B). The SNX27-knockdown HEK-293-HA-NHE3 cells that contained rat-derived SNX27–green fluorescent protein (GFP) effectively rescued NHE3 basal activity and surface expression (Figure 3, C and E).

We next determined whether the regulation of NHE3 activity by SNX27 requires its ability to bind the single SNX27 PDZ domain. PDZ domains generally bind to their ligands through a groove formed by the sequence GYGF (Doyle et al., 1996; Jemth and Gianni 2007). The binding ability of PDZ domains to most of their ligands is abolished in GYGF (aa 52–55) mutants (Karthikeyan et al., 2002; Weinman et al., 2003). Therefore the GYGF sequence was mutated to GAGA, and mutant GFP-SNX27-PDZ-GAGA was studied for its effect on NHE3 activity and cell surface expression. By immunolocalization, SNX27-GFP colocalizes prominently with EEA1-containing endosomes, as shown previously (Lauffer et al., 2010). Similar to wild type, GAGA mutant also localized to the EEA1 compartment (Figure 3F). Wild-type rat-GFP-SNX27 fully rescued basal NHE3 activity; cell surface expression of NHE3 was also similar to wild-type SNX27 in control cells, whereas the PDZ mutant rescued neither wild-type NHE3 activity nor cell surface expression (Figure 3, C and D). In addition, the PDZ mutant fusion protein
FIGURE 3: PDZ domain–mediated interaction of SNX27 maintains plasma membrane localization and activity of NHE3. (A) Western blot analysis of total cell lysate prepared from HEK293-HA-NHE3 cells infected with lentivirus control shRNA or SNX27 shRNA. GAPDH was used as internal control. (B) Densitometric analysis of total protein expression from control shRNA and SNX27 shRNA–infected lysates showed that SNX27 protein expression was significantly reduced in HEK293-HA-NHE3 cells. Results are means ± SE of four separate studies. p values are comparison between control shRNA and SNX27 shRNA. (C) Na⁺/H⁺ exchange was measured in HEK293-HA-NHE3 cells expressing SNX27 shRNA or control shRNA with or without reconstitution of SNX27 by cotransfection of rat SNX27-GFP or PDZ mutant SNX27-GFP-GAGA. Results are means ± SE of four separate studies. p values are comparison between control shRNA and SNX27 shRNA or GFP-SNX27 and GFP-SNX27-GAGA. (D) Western blot analysis of NHE3 surface expression using biotinylation in HEK-HA-NHE3 cells expressing SNX27 shRNA or control shRNA with or without replacement by cotransfection of rat SNX27-GFP or its PDZ mutant SNX27-GFP-GAGA. A representative blot from three independent experiments with similar results. (E) GST, GST-SNX27-PDZ, or GST-SNX27-PDZ-GAGA was mixed with HEK-HA-NHE3 cell lysate and then subjected to pull down with GSH resin. Samples were analyzed by Western blot (IB) with antibodies against HA and GST. The experiment was repeated three times, and one representative result is shown. (F) Representative examples of fluorescence localization patterns of rat GFP-SNX27 or its PDZ mutant (GYGF → GAGA) versions of SNX27 relative to EEA1, verifying that its PDZ domain is not required for early endosomal localization of SNX27. Bar, 10 μm. Representative images.
SNX27 regulates NHE3 trafficking

That the SNX27 knockdown reduced basal NHE3 activity and surface expression without a change in total cellular expression and that the intracellular pool of NHE3 partly overlaps with SNX27 in endosomes support that SNX27 plays a role in NHE3 trafficking. Mechanistic studies were undertaken to define this role of SNX27 in NHE3 recycling in polarized epithelial cells.
basal endocytosis was not altered by SNX27-KD in SK-CO15 cells; however, exocytic insertion of NHE3 to the plasma membrane was significantly reduced in the absence of SNX27. These studies suggest that SNX27 is required for delivery of NHE3 from endocytic vesicles to the plasma membrane.

Acute stimulation of NHE3 activity by serum is mediated by SNX27
Most of the acute stimulation of NHE3 involves an increase in surface expression, which is a result of an increase in exocytotic trafficking (Yang et al., 2000; Peng et al., 2001; Bobulescu et al., 2005; Zachos et al., 2005; Donowitz et al., 2009). Because exocytosis, but not endocytosis, was affected by KD of SNX27 in SK-CO15 cells, we next investigated whether the acute stimulation of NHE3 by dialedyzed serum, which involves an increase in surface expression, was also affected in SNX27-knockdown cells. Therefore, Na”/H” exchange activity and cell surface amount were measured in response to acute stimulation by serum.

NHE3 regulation. Because the surface amount of NHE3 is also dependent on the balance of exocytic insertion and endocytic internalization, we further examined whether these two processes are affected in SNX27-knockdown SK-CO15 cells. Cell surface biotinylation-based methods were used to determine the rate of exocytic insertion and endocytic internalization separately. The rate of exocytic insertion of NHE3 was measured for 5, 10, and 20 min in SNX27-KD SK-CO15 cells, as shown in Figure 5, A and B. The exocytic insertion of NHE3 was ∼50% less than (p < 0.05) that of the control cells at all the time points tested. Whereas the rates of exocytosis were significantly reduced in SNX27-KD cells, the rate of endocytosis in control and knockdown cells was not significantly different (Figure 5, C and D). The amount of NHE3 internalized at 5, 10, and 20 min was normalized to the surface NHE3 that was measured at 4°C and was not treated with glutathione (GSH; control). Taken together, the rate of NHE3 endocytosis was not significantly different between SNX27-control and SNX27-KD cells, showing that basal endocytosis was not altered by SNX27-KD in SK-CO15 cells; however, exocytic insertion of NHE3 to the plasma membrane was significantly reduced in the absence of SNX27. These studies suggest that SNX27 is required for delivery of NHE3 from endocytic vesicles to the plasma membrane.

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to serum for different time periods (15, 30, and 60 min) in SK-CO15 control and SNX27-KD cells. The basal rate of Na\(^+\)/pH change by NHE3 was measured in the presence of the NHE1 and NHE2 inhibitor Hoe-694 (50 μM). Serum treatment in control cells increased NHE3 activity by ∼47, 78, and 103% at 15, 30, and 60 min, respectively (Figure 6A). In contrast, the loss of SNX27 significantly inhibited the effect of serum on NHE3 activity, resulting in no significant stimulation at 15- and 30-min time points, whereas there was significant stimulation (∼80%) at 60 min (Figure 6A). These findings showed that SNX27 plays a significant role in, but is not obligatory for, serum-mediated acute stimulation of NHE3 activity. On the basis of the foregoing finding, we hypothesized that NHE3 requires SNX27 in order to maintain the rapid rate of trafficking to the plasma membrane. Therefore we next investigated the rate of exocytotic trafficking of NHE3 at different time points (15, 30, and 60 min) in response to serum in polarized SK-CO15 WT and SNX27-KD cells. In control cells, the exocytosis of NHE3 in response to serum starts as early as 15 min and gradually increases approximately twofold by 60 min (Figure 6B). In contrast, in SNX27-KD cells, the exocytosis of NHE3 in response to serum was slower than with control cells; the only significant amount of exocytosed NHE3 was at 60 min (Figure 6B). However, the percentage increase in NHE3 activity and surface amount at 60 min was similar in control and SNX27-KD cells. Taken together, these results demonstrate that the presence of SNX27 is required to maintain the rate of exocytotic trafficking of NHE3 in response to serum.

**Half-life of plasma membrane but not total NHE3 depends on SNX27 in polarized epithelial cells**

The phenotype of SNX27 depletion reduced NHE3 surface expression, as assessed by cell surface biotinylation. Plasma membrane retention of protein depends on the balance between the rates of endocytosis and exocytosis. Biochemical internalization and recycling assays revealed that although NHE3 was internalized at similar relative rates in SNX27-depleted cells, the return to the cell surface was severely inhibited (Figure 5, A and B). Therefore we investigated the half-life of NHE3 initially on the plasma membrane by surface biotinylation, as described previously (Cavet et al., 2001). We determined the total surface biotynlated NHE3 protein remaining with cells at 0–24 h after initial biotinylation of surface protein in control and SNX27-KD SK-CO15 cells. The amounts of biotynlated NHE3 protein left at different time periods were normalized to surface NHE3 at time 0 (cells always at 4°C). These results (Figure 7, A and B) demonstrated that plasma membrane NHE3 in control and SNX27-KD cells has different degradation rates. Whereas control cells had NHE3 plasma membrane half-life of ∼12.5 h, SNX27-KD cells had a much shorter plasma membrane half-life of ∼7.5 h (Figure 7B).

Although we did not notice any change in total NHE3 expression between control and SNX27-KD cells, to further confirm this observation, we performed degradation (half-life of total NHE3) assays from 0 to 24 h in SK-CO15 control and SNX27-KD cells treated with cycloheximide (100 μM) to block synthesis of new proteins. Cell lysates from different time points were separated by SDS–PAGE and transferred to nitrocellulose membranes. Figure 7, C and D, showed that even though the surface half-life was much shorter in SNX27-KD cells than in control, the half-lives of total NHE3 were not significantly different. This is not a cell type–specific but instead is a substrate-specific effect, as the other known cargoes of SNX27 in these cell types showed higher degradation of total protein expression in response to SNX27-KD (Figure 2F). These results show that SNX27 regulates the retention or stability of NHE3 on the plasma membrane.

**DISCUSSION**

Epithelial cells contain multiple PDZ proteins, which are localized to specific subcellular domains, including the brush border (BB), basolateral membranes, and tight junctions, as well as in the Golgi (CAL) and endosomes (sorting nexin 27; Cheng et al., 2002, 2004; Brone and Eggemont 2005). The major identified class of PDZ domain–containing proteins in the BB is the NHERF family, although the unrelated Shank2 is also present. These proteins regulate different aspects of transport protein function. Use of NHE3 as an example shows that the contributions of PDZ domain–containing proteins...
using an unbiased global analysis of SNX27-mediated sorting found that it affected >100 cell surface proteins, which include the glucose transporter GLUT1, the Menkes disease copper transporter ATP7A, zinc and amino acid transporters, and multiple signaling receptors (β2AR, TGFβ-receptor1, etc.). All of these proteins require the SNX27–retromer complex to prevent lysosomal degradation and maintain surface levels (Temkin et al., 2011; Steinberg et al., 2013).

These reports, including our present results, are different from the previously proposed roles of SNX27 in promoting endocytosis or lysosomal delivery of PDZ motif–bearing cargo (Joubert et al., 2004; Lunn et al., 2007). The possible explanation is that the previous studies relied entirely on SNX27 overexpression, which is known to produce additional effects consistent with expression-dependent differences in the functional effects of other sorting nexins (Carlton et al., 2005). To avoid the additional effects caused by the overexpression system, we chose a model that contains endogenous NHE3 and SNX27 proteins. Here we demonstrated with shRNA-mediated knockdown and subsequent rescue experiments that loss of SNX27 in parallel decreased the apical localization and basal activity of NHE3 in intestinal epithelial cells. In addition, the change in activity and surface expression depends on the PDZ domain of SNX27. This conclusion was reached because the reduced NHE3 activity and surface expression that resulted from knockdown of

![FIGURE 7: Half-life of plasma membrane but not total NHE3 depends on SNX27. (A) Plasma membrane NHE3 protein in control and SNX27 shRNA–expressing cells was biotinylated at 4°C using NHS-SS-biotin. Cells were incubated at 37°C and harvested at various time points. After solubilization, biotinylated protein was recovered with streptavidin–agarose beads and analyzed by Western blotting as described. A single representative blot of three independent experiments with similar results. (B) Densitometric analyses of Western blots. The amount of biotinylated NHE3 protein left at different time periods was normalized to surface NHE3 at time 0 (cells always at 4°C). Error bars represent the SE of four experiments. *p < 0.05 values are comparison between control and SNX27-depleted cells. (C) Total NHE3 levels in control and SNX27-depleted cells treated with cycloheximide (100 μM) over the indicated periods. (D) Densitometric analyses of Western blots were performed to calculate percentage of NHE3 remaining over indicated time periods. A representative blot for four independent experiments is shown. Error bars represent the SE of four experiments.](image-url)
SNX27 was reversed by knocking-in wild-type SNX27-rat, whereas the PDZ-domain mutant of the same construct was unable to duplicate the rescue. Our results are in agreement with a previous detailed study showing that SNX27 is a critical sorting protein for PDZ motif–directed endosome-to–plasma membrane traffic of the β2ARs (Lauffer et al., 2010; Loo et al., 2014).

In confluent polarized monolayers, NHE3 is localized at the apical and subapical region, where it is present in multiple pools (Li et al., 2001; Cha et al., 2004; Alexander et al., 2005). These pools are rapidly activated or inhibited by changes in trafficking, which probably mimics renal and intestinal physiology (Cha et al., 2004; Alexander et al., 2005). Given that SNX27 is known to localize to early endosomes (Lauffer et al., 2010), we hypothesized that NHE3 might transiently interact with SNX27 during intracellular trafficking. Using tagged NHE3 protein as well as antibody staining of native proteins following endocytosis, we showed overlap of the two proteins on the EEA1-containing early endosome. NHE3 traffics in multimeric complexes that are regulated by PDZ-domain protein interactions involving NHERF proteins (Cha et al., 2004; Cha et al., 2010). Thus it is likely that there are other proteins that interact with the SNX27-NHE3 complex to regulate endosomal trafficking. Analysis of all proteins in the SNX27-NHE3 complex and an examination of multiple endosomal pools of NHE3 proteins will be required to fully understand the role of SNX27 in regulating NHE3 activity.

Under basal conditions, NHE3 cycles between the plasma membrane and the endosomal recycling compartment (D’Souza et al., 1998; Kurashima et al., 1998; Janecki et al., 2000a,b; Hu et al., 2001; Akhter et al., 2002). As part of digestive physiology, NHE3 is acutely stimulated and inhibited by changes in trafficking, and we previously identified the role of some PDZ-domain proteins under different physiological conditions (Zachos et al., 2009; Cha et al., 2010; Yang et al., 2013). However, our previous studies focused on the role of PDZ proteins in cytoskeleton association of NHE3. In contrast, in the present study, we explored the role of the PDZ-domain protein SNX27 in NHE3 trafficking in detail. Biochemical internalization and recycling assays revealed that although NHE3 was internalized at similar relative rates in SNX27-depleted cells, the return to the cell surface was severely inhibited by knocking down SNX27. This is in accordance with other reports showing PDZ domain–mediated protein interactions in determining the endomembrane trafficking for several membrane proteins (Lin and Huganir 2007; Cushing et al., 2008; Lauffer et al., 2010). Surprisingly, depletion of SNX27 decreased the stability of NHE3 on the plasma membrane but not the stability of total NHE3, as confirmed by degradation assays in cells treated with cycloheximide to block protein synthesis. Unlike other proteins regulated by SNX27 (e.g., Glut-1 and β2AR), this finding is unique to NHE3. There could be two possible explanations for this finding: 1) other FERM-like domain–containing proteins in early endosomes mediate retrieval of NHE3 from the lysosomal pathway in absence of SNX27, and/or 2) the SNX27-dependent exocytic pool, which has the same half-life as the total NHE3 pool, is stabilized by another SNX27-dependent protein, perhaps one the exocytosis of which also requires SNX27. In the absence of this additional protein, surface NHE3 stability/surface half-life is reduced. This pool of NHE3 is exocytosed rapidly in response to serum, since by 60 min after serum stimulation, the NHE3 activity is no longer dependent on the presence of SNX27. However, further studies are needed to understand this process in detail.

Several apical-domain pools of NHE3 in epithelial cells have been defined previously. These include NHE3 in lipid rafts and outside lipid rafts and NHE3 bound to megalin (with heavier density and less activity) or not bound to megalin (lighter density and more activity). Intracellular NHE3 also exists in two pools, one of which is rapidly exocytosed, whereas the other moves to the apical membrane with much slower kinetics (Alexander et al., 2005, 2007; Alexander and Grinstein 2006). Serum-stimulated exocytosis of NHE3 had two phases, with loss of stimulation with SNX27KD only at shorter time points (<60 min). On the basis of our results and previous reports, we believe that SNX27 plays a major role in trafficking of NHE3 from an intracellular pool that responds to stimuli to increase NHE3 by rapid exocytosis rather than the pool that moves more slowly and that both pools are involved in stimulation of NHE3 activity by serum.

Vesicle membrane trafficking and recycling is important to maintain proper transporter function in polarized epithelial cells. Our data established the role of a PDZ-domain protein (SNX27) that is directly involved in cellular trafficking and hence in maintaining normal physiology in epithelial cell. Collectively the results of the present study provide several lines of evidence indicating that in epithelial cells, SNX27 has a necessary role in PDZ motif–directed exocytosis of membrane proteins and that it mediates this sorting function directly from the early endosome. This finding sheds light on the concept of motif-directed molecular sorting in the recycling pathway and supports its physiological significance.

**MATERIALS AND METHODS**

**Materials**

Glutathione Sepharose 4B resin was from GE Healthcare Life Science (Pittsburgh, PA). Amylose resin and rabbit anti-MBP were from New England Biolabs (Ipswich, MA). BECF-AM, nigericin, and Hoechst 33342 were from Life Technologies (Grand Island, NY). Mouse anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mouse anti-actin were from Sigma-Aldrich (St. Louis, MO). Rabbit anti-GFP, Alexa Fluor 488– and 568–conjugated goat anti-mouse and anti-rabbit secondary antibodies, and Alexa Fluor 568–conjugated phallolidin were from Life Technologies. Mouse anti-HA was from Covance (Princeton, NJ). Mouse anti-GST was from Cell Signaling Technology (Danvers, MA). IRdye-700– and IRdye-800–conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were from Rockland Immunochemicals (Gilbertsville, PA) and were used with the Odyssey system (LI-COR, Lincoln, NE) for Western blot analysis. Mouse anti-GFP, mouse anti-SNX27, rabbit anti-β2AR, and rabbit anti-GLUT-1 were from Abcam (Cambridge, MA).

Plasmid pCDNA3.1-HA-NHE3 was constructed previously (Murtazina et al., 2006). To study the role of the C-terminal-end putative I PDZ binding motif (β2AR) of NHE3, we made HA-NHE3ΔC4 in pCDNA3.1/Hygro+vector with HindIII/XbaI cloning site. HA-NHE3ΔC4 (HA-NHE3 with deleted last four amino acids) was made by PCR using sense primer ATAAAGCTTGTAGTCAAGGGGCCGGGGGG and antisense primer GCTCTAGATCCTCGGGGTGTTCAGCGCC. Anti-NHE3 polyclonal antibodies were raised previously against a synthetic peptide corresponding to amino acids 809–831 (NH2-DSFLQADGHEEQLQPAAPESTHM-GAGA) point mutation was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

**Cell culture and construction of SNX27-knockdown cells**

Rat SNX27b (SNX27b) cDNA was kindly provided by Jae Cheng (Johns Hopkins University, Baltimore, MD). The SNX27b PDZ (GYGF → GAGA) point mutation was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).
following the manufacturer’s instructions using primers 5′-AAGTCCC-GAACTCGGCGCCGGCGCCACGTTGCGGCGCGCCA-3′ and 5′-TT-GGCCCCCGCAGTTGGCGCGCCGCGGATTCGACT-3′. cDNAs of human GFP-Rab4, Rab5 and Rab11 were kindly provided by James Goldenring (Vanderbilt University School of Medicine, Nashville, TN). Human embryonic kidney 293 cells and human intestinal polarized epithelial cell lines Caco-2BBE and SK-CO15 (kindly provided by Asma Nusrat, Emory University, School of Medicine, Atlanta, GA) were used for these studies. HEK293T cells were used to generate HA-NHE3 stably expressing cells by transient transfection of pcDNA3.1-HA-NHE3 with G418 selection. Stable HEK293-HA-NHE3 cells were cultured in DMEM (Invtrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (50 μm/l), streptomycin (50 μg/ml), and G418 (1000 μg/ml) at 37°C with 5% CO2 and 95% humidity. SK-CO15 cells were grown on membranes (Transwells or filters; Corning, Corning, NY; Zachos et al., 2009) and maintained in DMEM supplemented with 10% FBS, penicillin (50 μg/ml), streptomycin (50 μg/ml), 1 mM sodium pyruvate, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1% nonessential amino acids. Stable SNX27 knockdown (SNX27-KD) cells (SK-CO15 and HEK-293) were generated using lenti-shRNA constructs. In brief, gene sequence–specific shRNA clones were constructed within the lentivirus plasmid vector pJKO-1-purovirin (Sigma-Aldrich). Three constructs were tested to generate lentiviral transduction particles: A: CCAGGCGCCATTCGTTACATCTCGAGATGTA-TACCTGTATGACAGCGCT-3′  
B: CCAGGCGCCATTCGTTACATCTCGAGATGTA-TACCTGTATGACAGCGCT-3′  
C: CCAGGCGCCATTCGTTACATCTCGAGATGTA-TACCTGTATGACAGCGCT-3′  

The production of lentiviral particle and lentiviral transduction was done as described previously (Zachos et al., 2009; Sarker et al., 2011; Yang et al., 2014). Stable cell lines of SK-CO15 and HEK293 with expression of SNX27 knocked down were generated by infecting cells with the lentiviral vectors, and selection was achieved by inclusion of 2 and 1 μg/ml puromycin for SK-CO15 and HEK-293-HA-NHE3 cells, respectively, in the culture medium. Knockdown of protein expression was verified by Western blot analysis. SNX27 shRNA construct C was ineffective in reducing endogenous SNX27 expression in SK-CO15 cells, whereas constructs A and B knocked down endogenous SNX27 expression in SK-CO15 cells by ~80% and ~50%, respectively. Construct A was used to knock down SNX27 in SK-CO15 and HEK-293-HA-NHE3 cells. As a transduction control, cells were transduced with a lentivirus plasmid vector containing shRNA that does not match any known human gene (Sigma-Aldrich). Infected cells were maintained under selection pressure of puromycin.

Expression of recombinant proteins in bacteria and purification cDNAs encoding rat SNX27b were generated using the following primer pairs:

SNX27-FL (aa 1–539): 5′-CGCAAGGGATCCATGCGCCGAC-GAGGACGGG-3′ and 5′-GAAAGGGATTCTCAGTGGG-CCCATCCCT-3′  
PDZ (aa 1–156): 5′-CGGACACGATCCATGCGCCGAC-GAGGACGGG-3′ and 5′-CTTTCGAAATCTATGTGTAAT-CATAAA-3′  
FERM (aa 272–539): 5′-GTTGTCGATCCATGCTGGA-GCTGAGA-3′ and 5′-GAAAGGGATTCCTAGTGGGC-CACATCCCT-3′  
PDZ+PX (aa 1–266): 5′-CGCAAGGGATCCATGCGCCGAC-GAGGACGGG-3′ and 5′-ATTGAGAATTTCAATTCCTCAG-GAGATCTGA-3′  
PX+FERM (aa 158–539): 5′-TACACAGATTTGAAAGG-ACCGAAGCT-3′ and 5′-GAAAGGGATTCCTAGTGGGC-CACATCCCT-3′  

These constructs were cloned into the BamHI-EcoRI site of pGEX-4T-1 vector.

NHE3 recombinant protein expression and purification cDNA fragments encoding rabbit NHE3 carboxyl-terminus fragment 642C (amino acids P642–M832) were amplified by PCR and inserted into the previously constructed vector pMBP (Yang et al., 2014) between BamHI and Xhol.

Expression constructs were transformed into BL21(DE3) strain (EMD Millpore, Billerica, MA). When the bacterial culture reached an OD of ~0.8, protein expression was induced with 0.3 mM isopropyl-β-D-thiogalactoside at 16°C overnight. MBP-tagged proteins were purified with a column packed with amylose resin by gravity flow following the New England Biolabs manuals of the pMAL system. GST-tagged protein was purified in a gravity-flow column following the instructions from GE Healthcare. Purified proteins were concentrated by Amicon Ultra-15 Centrifugal filter units (EMD Millipore), supplemented with 10% glycerol and 10 mM dithiothreitol, and stored at −80°C.

Coimmunoprecipitation Coimmunoprecipitation experiments were performed using lysates from Caco-2BBE cells infected with HA-NHE3 adenovirus (Sarker et al., 2011). Cell lysates were prepared in lysis buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM EDTA trisodium, 3 mM lysis buffer ethylene glycol tetraacetic acid, 1 mM Na2VO4, and 1% Triton X-100 with protease inhibitor cocktail; Sigma-Aldrich). Anti-HA affinity matrix was washed with this buffer three times. Aliquots (2 mg of protein) of lysate were incubated with 15 μl of monoclonal anti-HA affinity matrix at 4°C for 4 h on a rotating shaker. Beads were washed five times with the same buffer and eluted with 2.5x Laemmli buffer. The input and eluted samples were separated by SDS–PAGE and transferred to nitrocellulose membranes. The immunoblots were probed with polyclonal anti-HA or monoclonal anti-SNX27 primary antibodies, followed by fluorescently labeled secondary antibody (IRDye 800 or Alexa Fluor 680) according to the manufacturer’s protocol, and bands were visualized by the Odyssey system.

GSH-resin pull down For interaction studies, 1 nmol of recombinant GST-tagged protein was used as bait for pull downs. As prey, 1 nmol of purified MBP-tagged recombinant protein or 1 mg of cell lysate was used as indicated. The volume of the final mixture was adjusted to 500 μl with the lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na2VO4, 0.5% Triton X-100, and protease inhibitors). GSH-resin (Glutathione Sepharose 4B resin) was washed with lysis buffer.
three times. Each bait–prey mixture was mixed with 10 μl of resin and incubated at 4°C for 3 h on a rotating shaker. Resin was washed with the same lysis buffer four times and then eluted with lysis buffer supplemented with 10 mM glutathione. The input and elution samples were analyzed by SDS–PAGE and Western blotting.

**Immunofluorescence**

SK-CO15 cells were grown on Transwell filters (Corning) until 6–7 d postconfluence. Triple-HA-tagged rabbit NHE3 in replication-deficient adenovirus (Sarker et al., 2011) was transiently infected into SK-CO15 cells for endocytosis-based colocalization analysis. transiently transfected SK-CO15 cells were incubated with 5 μg/ml HA or GFP antibody with 10% FBS for 1 h at 4°C. After binding, cells were incubated for 1 h at 37°C. The cells were then washed in ice-cold phosphate-buffered saline (PBS), and surface-bound antibody was stripped from the cells with cold PBS, pH 2.5, for 2 × 1 min. After the acid rinse, cells were washed once in PBS and fixed in cold 4% parafomaldehyde for 20 min, blocked with 1% bovine serum albumin (BSA) in PBS for 10 min, and incubated with anti-mouse Alexa Fluor 594– and anti-rabbit Alexa Fluor 488–conjugated secondary antibodies (1:100) in 0.1% saponin (Sigma-Aldrich) in PBS with Hoechst dye for 1 h at room temperature. Cells were washed three times with PBS and mounted with Gel Mount (Sigma-Aldrich) and then examined with a Zeiss LSM510 confocal fluorescence microscope. Results were from six to eight individual experiments.

HEK-293-HA-NHE3 control or SNX27-KD cells were grown on tissue culture coverslips to ~60% confluence. These cells were then transfected with GFP-Rab4, Rab5, Rab11, SNX27wt, or SNX27-GAGA (PDZ2 mutant) using Lipofectamine 2000 (Life Technologies). At 1 d posttransfection, cells were fixed with 3% paraformaldehyde/PBS for 20 min at 4°C, and the residual formaldehyde was neutralized with 20 mM glycine in PBS for 10 min. Cells were then permeabilized for 30 min in 0.1% saponin/PBS before being blocked for 30 min in 1% BSA/PBS supplemented with 10% FBS. Cells were incubated with respective primary antibody in 1% BSA/PBS for 60 min at room temperature. After three 10-min washes in 0.1% saponin/PBS, goat secondary antibodies (Alexa conjugates; Invitrogen) were added at 1:100 dilutions in 1% BSA/PBS, incubated for 30 min, and again washed three times for 10 min in 0.1% saponin/PBS. Cells were then mounted with Gel Mount and viewed on a Zeiss LSM510 confocal fluorescence microscope.

**Measurement of Na+/H+ exchange activity**

Cellular Na+/H+ exchange activity in HEK293-HA-NHE3 or SNX27-KD cells grown to ~70% confluence on glass coverslips and post-confluent SK-CO15 cells grown on Transwell filters was determined fluorometrically using the intracellular pH-sensitive dye 2′,7′-bis(carboxyethyl)5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM, 5 μM) as described previously (Levine et al., 1995). HEK-293 cells were exposed to 40 mM NH4Cl for 15-min dye loading, and SK-CO15 cells were exposed to 50 mM NH4Cl for the final 10 min of the 50-min dye loading period. Endogenous NHE1 in HEK-293 cells and NHE1 and NHE2 activity in SK-CO15 cells were inhibited by 10 or 50 μM HOE694, respectively, present in all solutions. At the end of each experiment, the fluorescence ratio was converted to pH, using the high–potassium/nigericin method (Levine et al., 1993). The initial rate (approximately the first minute) of NHE3 activity was quantitated and expressed as change in pH/minute (Watson et al., 1991; Janecki et al., 1998) Mean ± SE was determined from four separate experiments.

**Cell surface biotinylation and immunoblotting**

Postconfluent (6–7 d) control and SNX27-KD SK-CO15 cells were grown on 10-cm-diameter filters (Corning). The cells were then serum starved for ~3 h. All subsequent manipulations were performed at 4°C. For surface labeling of NHE3, cells were incubated with 1.5 mg/ml NHS-SS-biotin (biotinylation solution; Pierce Chemical, Rockford, IL) for 20 min and repeated once, solubilized with lysis buffer, and then incubated for 4 h with streptavidin-agarose beads. Western analysis and the quantification of the surface fraction were performed as described previously (Akhter et al., 2002; Singh et al., 2014a).

**Endocytosis internalization and exocytic insertion assays**

Endocytosis was measured by a protocol slightly modified from the reduced glutathione (GSH)–resistant endocytosis assay described previously by us (Lin et al., 2011b). Briefly, 6 d postconfluence, polarized SK-CO15 control and SNX27-knockdown cells were serum starved for 3 h and labeled with 1.5 mg/ml sulfo-NHS-SS biotin for 30 min at 4°C (this was repeated once), and then nonbound biotin was quenched as described. Cells were rinsed with PBS at 37°C and then incubated in serum-free medium at 37°C for 0, 5, 10, 15, or 20 min. Cells were rinsed with ice-cold PBS twice. Surface biotin was cleaved with GSH-containing buffer (150 mM GSH, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.8) for 5 min. The biotinylated NHE3 that was endocytosed was protected from cleavage by GSH. Cells were then solubilized in lysis buffer, biotinylated proteins were retrieved with streptavidin beads, and internalized NHE3 was assayed as described via Western analysis and normalized to the surface NHE3 initially present.

To measure exocytic insertion of NHE3 in basal conditions, confluent serum-starved SK-CO15 control and SNX27-knockdown cells were rinsed with PBS-Ca-Mg two times at 4°C. Surface proteins accessible to NHS-SS-biotin were masked by reaction with membrane-impermeant NHS-acetate (1.5 mg/ml) at 4°C for a total time of 2 h, adding fresh NHS-acetate every 40 min. Then cells were incubated with quenching buffer (100 mM glycine in PBS-Ca-Mg) twice with gentle shaking for a total time of 20 min at 4°C. After quenching, cells were incubated with prewarmed serum-free medium for 5, 10, 20, and 30 min at 37°C. One set of cells treated with sulfo-NHS-acetate at 4°C but never warmed to 37°C served as the zero time point. Cells were then labeled with sulfo-NHS-SS-biotin (1.5 mg/ml) and treated with lysis buffer as described. The biotinylated fraction was precipitated with avidin-agarose beads. The resultant precipitate was subjected to SDS–PAGE, and biotinylated NHE3 was detected by quantitative Western blot analysis as described. Biotinylated protein was normalized to the total amount of NHE3 present in the lysate at each time point and expressed as percentage exocytosed.

**Measurement of half-life of plasma membrane NHE3 using cell surface biotinylation**

To determine the half-life of plasma membrane NHE3 in wild-type and SNX27-KD cells, a cell surface biotinylation method was used, as described previously (Cavet et al., 2001). The purpose of this experiment was to determine the stability of NHE3 on the cell surface in the presence and absence of SNX27.

**Total NHE3 degradation assay**

Postconfluent control and SNX27-KD SK-CO15 cells were grown on Transwell filters and treated with 100 μM cycloheximide for the indicated time points. Cells were lysed in PBS with 1% (vol/vol) Triton X-100, and NHE3 levels were determined by quantitative Western
blotting. β-Actin fluorescence intensity was used to normalize the detected levels of NHE3. The untreated controls were calculated as the percentage of untreated control for each time point.

Statistical analysis
Experiments were repeated at least four times. Results are presented as means ± SE. Comparisons were performed by unpaired Student’s t tests or analysis of variance for multiple comparisons.

ACKNOWLEDGMENTS
This work was supported in part by National Institutes of Health Grants P01DK089502 to the Conte Hopkins Digestive Diseases Basic and Translational Research Core Center.

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