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IRBIT plays an important role in NHE3-mediated pH_i regulation in HSG cells

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ABSTRACT

Expression of inositol-1,4,5-trisphosphate (IP_3) receptor-binding protein (IRBIT) has been reported in epithelial cells. However, its role in pH_i regulation is not well understood. In this study, we investigated the role of IRBIT in pH_i regulation, mediated by Na^+/H^+ exchangers (NHEs), in salivary glands. We measured pH_i recovery from cell acidification in BCECF-loaded salivary HSG cells. Western blot and co-immunoprecipitation (CO-IP) assays were also performed, showing that NHE1, 2 and 3 are expressed, and IRBIT binds to NHE3. HOE642, a specific NHE1 blocker, inhibited pH_i recovery, but 40% pH_i recovery was still observed even at the highest concentration of HOE642. Furthermore, pretreatment of the cells with siIRBIT significantly inhibited pH_i recovery, indicating that NHE3 potentially plays a role in pH_i recovery as well. The amount of membrane-localized NHE3 and its interaction with IRBIT are also significantly increased by cell acidification. In addition, we found that Ste20p-related proline alanine-rich kinase (SPAK) reverses the effect of IRBIT on membrane NHE3 translocation. Taken together, we conclude that IRBIT plays an important role in pH_i regulation, mediated by NHE3, and further regulated by SPAK.

1. Introduction

Inositol-1,4,5-trisphosphate (IP_3) receptor-binding protein (IRBIT) was discovered in a search for proteins which interact with the IP_3-binding domain of IP_3 receptors [1]. IRBIT competes with IP_3 for interaction with the NH_2-terminal domain of the IP_3 receptor and inhibits the receptor function [2]. IRBIT was also reported to interact with and regulate the activity of the sodium bicarbonate transporter NBCe1-B as its primary target molecule [3]. Since then, there have been many studies elucidating the mechanism by which IRBIT regulates the Na^+-HCO_3^- cotransporter family [3–5].

Intracellular pH (pH_i) is finely controlled in mammalian epithelial cells, and many membrane transporters, including Na^+/H^+ exchangers (NHEs), bicarbonate transporters and anion exchangers, are involved in the pH_i regulatory process [6]. In salivary gland tissues, four kinds of NHE subtypes, NHE1 to NHE4, have been reported [7]. NHE1 localizes at the basolateral membrane is ubiquitous in epithelial cells, functioning as a housekeeper of cytoplasmic pH [8–10]. NHE2 and NHE3 are expressed at the luminal membrane of the interlobular and excretory ducts in mouse and rat salivary glands, whereas NHE4 is found basolaterally distributed in acinar and duct cells in rats [7,11]. The function of NHE2 and NHE4 is not yet clear [7,10]. NHE3 functions in the luminal membrane of the pancreatic duct [12] and is thought to be involved in transepithelial NaCl reabsorption [7,13,14]. NH3 could be a part of a HCO_3^- regulating complex in the duct, which serves a HCO_3^- salvage mechanism that maintains acidified saliva in the resting state [10]. However, the regulatory mechanism of NHE3 has not been rigorously studied.

Ste20p-related proline alanine-rich kinase (SPAK) is a serine/threonine (S/T) kinase with putative regulatory domains located downstream from its N-terminal catalytic domain [15]. Previous studies reported that SPAK regulates the functional expression of the NBCe1-B transporter [4,5,16]. SPAK phosphorylated basolateral NBCe1-B to reduce its surface expression and thus its activity [5,16].

In the present study, we examine whether IRBIT regulates NHE activity in salivary epithelial cells. We found that IRBIT plays a role in pH_i recovery from the cell acidification mediated by NHE3 in HSG cells. We also revealed that SPAK binds to NHE3 and IRBIT, subsequently regulating NHE3 activity.

2. Materials and methods

2.1. Reagents

IRBIT siRNA (siIRBIT) was purchased from Sigma Aldrich (St. Louis, MO, USA). SPAK siRNA (siSPAK) and the SPAK overexpression

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construct were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). NHE1–3 and IRBIT antibodies were purchased from Abcam Inc. (Cambridge, MA, USA), and the SPAK antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). HOE642, a specific NHE1 inhibitor [17], was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Membrane fractionation and western blotting

HSG cells were washed and sonicated in ice-cold 20 nM HEPES solution containing 1 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 0.3 mM phenyl methylsulfonyl fluoride at pH 7.4 (3 × 30 s, Branson Sonifer). The samples were incubated on ice for 20 min and subsequently centrifuged at 600*g* at 4 °C. The supernatants were further centrifuged at 20,000*g* at 4 °C. Original samples, the pellets (membrane fractions) and the corresponding supernatants (crude cytosolic fractions) were subjected to SDS/PAGE and immunoblotting. The protein samples were transferred to polyvinylidene difluoride membranes (0.45 μg, Millipore) and probed with polyclonal antibody specific for Na^+/H^+ exchanger 3 (NHE3) at a dilution of 1:1000, as well as polyclonal antibody specific for G_β at a dilution of 1:500. Anti-rabbit IgG was used as a secondary antibody followed by ELC detection of the corresponding protein bands. G_β, a subunit of G-protein in GPCR signaling, was used as a quantitative control for plasma membrane preparation [18].

2.3. Co-immunoprecipitation

HSG cells were washed twice in cold phosphate-buffered saline (PBS), scraped, and lysed in lysis buffer: RIPA buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 μg/mL Aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM Na_3VO_4, and 1 mM NaF for 15 min on ice, and were ultrasonicated 9 times for 10 s each. The lysates were then centrifuged at 13,000 rpm for 20 min. Protein concentrations were determined using the BCA protein assay kit (Pierce, IL, USA). Lysates (800 μg) were incubated with antibody at 4 °C overnight. The lysates were then incubated with 30 μL of Protein A/G Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight, followed by 20 washes with lysis buffer. All of the above steps were performed at 4 °C or on ice. The lysates were subsequently subjected to western blotting. Briefly, after western blotting transfer, the nitrocellulose membrane was washed three times in TBS-Tween buffer and the blot was developed according to standard procedures.

2.4. Treatment with siRNA

Following a 3-h starvation, HSG cells were transfected with siIRBIT at concentrations of 25 pmol and 50 pmol, with 50 pmol scrambled siRNA as a control, all of which were diluted in 250 μL of Dulbecco's Modified Eagle's Medium (DMEM). 10 μL Genefectine was diluted in 250 μL of the same medium. The diluted siRNA and Genefectine were mixed and added to the dish after 15 min. The final scrambled siRNA concentration was 10 nM, and the final siIRBIT concentrations were 5 nM and 10 nM. After 24 h, the medium was replaced with fresh DMEM containing serum.

2.5. Intracellular pH (pH_i) measurement

HSG cells were loaded with 2 μM 2′, 7′-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Molecular Probes, Inc., Eugene, OR) by adding stock solution to the culture medium, followed by incubation for 30 min. BCECF-AM was prepared from stock solution in DMSO; the final concentration of DMSO was 0.1% during loading. The fluorescence of BCECF-AM-loaded HSG cells was measured using a Photon Technology System (South Brunswick, NJ). Cells were perfused with HEPES-buffered solution: 10 mM D-Glucose, 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4. Acidification of the cells was induced by exposure to 20 mM NH_4Cl for 1 min in Na^+-free bath solution. During the pH_i measurements, bath solution was superfused at a flow rate of 3 mL/min. The Na^+/H^+ exchange rate was described by determining the slope along the early stages of pH_i recovery. The Na^+-dependent pH_i recovery rate was measured from acutely acidified cells using the NH_4Cl pulse technique in HCO_3^- free, HEPES-buffered bath solution.

2.6. Statistical analysis

All results are presented as a mean ± S.E., n = the number of experimental repeats with individual HSG cells, unless otherwise stated. Statistical significance was calculated using the Student's unpaired t test. A probability below 0.05 ($^*p < 0.05$) was considered significant.

3. Results

3.1. Subtypes of NHEs and their interactions with IRBIT in HSG cells

We first examined what types of NHEs are expressed in HSG cells using western blot analysis. All NHE subtypes measured, 1, 2 and 3, were expressed at the protein level. Strong single bands of 91 kDa, 92 kDa and 93 kDa corresponding to NHE1, NHE2 and NHE3 were detected in the plasma membrane and in cytosol (Fig. 1A). Expression of endogenous IRBIT was previously reported in HSG cells [19]. Thus, we next examined whether endogenous IRBIT interacts with any of the NHE subtypes using a co-immunoprecipitation (CO-IP) assay. IRBIT did not interact with NHE1 or NHE2, but did interact with NHE3 (lower panel in Fig. 1B).

3.2. The role of NHE3 in pH_i regulation

We next examined whether NHE3 plays a role in pH_i recovery in HSG cells. Pretreatment of cells with 5 μM or 10 μM HOE642, a

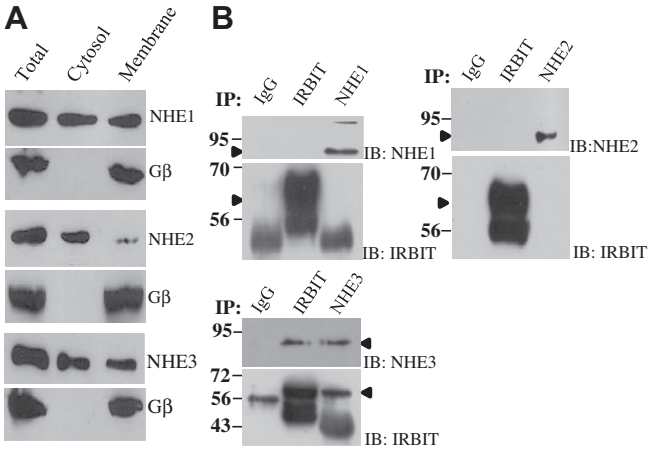

Fig. 1. Expression of the Na^+/H^+ exchangers NHE1, 2 and 3, and their interactions with IRBIT in HSG cells. (A) Western blot analysis probed with anti-NHE1, anti-NHE2 and anti-NHE3 antibodies. G_β, a G-protein subunit in GPCR signaling, was used as a quantitative control for plasma membrane preparation. The results are representative of three independent experiments. (B) HSG cells were subjected to co-immunoprecipitation (IP) with mouse IgG (control), anti-IRBIT, anti-NHE1, anti-NHE2, and anti-NHE3 antibodies (indicated by arrow heads), and the IPs were subjected to western blotting. The results are representative of three independent experiments. Mouse IgG was used as a negative control.

specific NHE1 inhibitor, significantly reduced rates of pH_i recovery by ~25.5% (0.008 pH unit/min ± 0.001, mean ± S.E., $n = 3$) and by ~55.3% (0.004 ± 0.001, $n = 3$), respectively. However, ~40% of pH_i recovery was still observed even at the higher concentration of HOE642 (50 μM) indicating that, aside from NHE1, other subtypes of NHEs, possibly NHE2 and/or NHE3, are involved in pH_i recovery (Fig. 2A).

We further examined the role of NHE3 in pH_i recovery by silencing endogenous IRBIT expression using siIRBIT. Two different concentrations of siRNA construct, 5 nM, and 10 nM, significantly reduced endogenous expression of IRBIT, by ~49%, and ~75%, respectively (Fig. 2B). Silencing of IRBIT by siIRBIT (5 nM, 10 nM) also significantly reduced the pH_i recovery rate by ~22.1% (0.009 ± 0.003, $n = 4$) and ~35.71% (0.007 ± 0.001, $n = 4$), respectively, in a concentration dependent manner (Fig. 2C) compared with the control group treated with scrambled siRNA (10 nM).

3.3. The effect of IRBIT on the translocation of NHE3

Since siIRBIT reduced NHE3-mediated pH_i recovery, we next examined whether IRBIT affects NHE3 translocation from the cytosol to the plasma membrane during cell acidification. Following NH_4Cl pulse, membrane expression levels of all NHE subtypes, 1, 2 and 3, are increased by ~54%, ~16%, and ~49%, respectively (2nd lanes in Fig. 3A) compared to the controls (1st lanes in Fig. 3A). Under the same conditions, silencing of endogenous IRBIT did not affect the membrane expression levels of NHE1 and 2 (3rd lanes in the upper and middle panel in Fig. 3A). However, siIRBIT significantly reduced the amount of membrane-localized NHE3 by ~34% (3rd lane in the lower panel in Fig. 3A), indicating that siIRBIT inhibits the expression level of NHE3 at the membrane. We further examined whether an interaction of IRBIT with NHE3 is substantially increased during cell acidification using a CO-IP assay. As shown in Fig. 3B, the amount of IRBIT co-precipitating with NHE3 was significantly increased, by ~49% ($n = 3$, 2nd lane in Fig. 3B), compared to the control (1st lane in Fig. 3B).

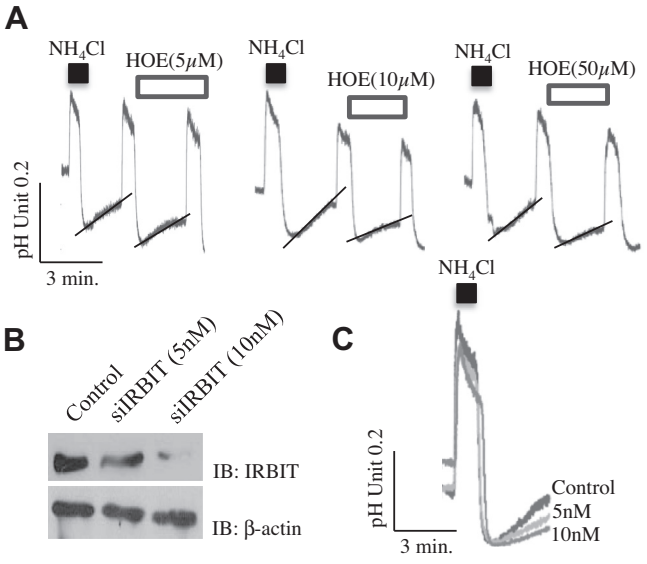

Fig. 2. The role of NHE3 in pH_i regulation in HSG cells. (A) The effect of HOE642 treatment on the Na^+-dependent pH_i recovery rate after a 20 mM NH_4^+ pulse (*black bars*). 5 μM and 10 μM HOE642 treatment significantly reduced pH_i recovery rate by ~25.5% (0.008 pH unit/min ± 0.001, mean ± S.E., $n = 3$) and ~55.3% (0.004 ± 0.001, $n = 3$), respectively. ~40% of pH_i recovery was still observed even at the highest concentration of HOE642, 50 μM. (B) Two different concentrations of siRNA construct, 5 nM, and 10 nM, significantly reduced endogenous expression of IRBIT by ~49%, and ~75%, respectively. (C) Representative pH_i recovery after pretreatment with scrambled (control) or two different concentrations of IRBIT siRNA. Silencing of IRBIT by treatment with siIRBIT at 5 nM or 10 nM significantly reduced the pH_i recovery rate by ~22.1% and ~35.71%, respectively, compared with the control, which was pretreated with scrambled siRNA.

3.4. The inhibitory effect of SPAK on IRBIT-induced NHE3 translocation

We finally examined whether NHE3 translocation is also regulated by Ste20p-related proline alanine-rich kinase (SPAK) using a CO-IP assay. We found that SPAK interacted with both NHE3 and IRBIT (indicated by arrow head in Fig. 4A and B). To examine the regulatory role of SPAK on the NHE3 translocation, we performed membrane fractionation assay, and measured pH_i recovery in SPAK-silenced cells in HCO_3^- free, HEPES-buffered bath solution. Interestingly, silencing of endogenous SPAK by ~20% (3rd lane in Fig. 4C) increased the amount of membrane-localized NHE3 by ~49% (1st lane in Fig. 4C), and enhanced NHE-mediated pH_i recovery by ~16% (0.010 ± 0.003, $n = 3$) (Fig. 4D) as compared with the control. Furthermore, overexpression of endogenous SPAK by ~25% (3rd lane in Fig. 4C) decreased the amount of membrane-localized NHE3 by ~21% (1st lane in Fig. 4C), and inhibited NHE-mediated pH_i recovery by ~18% (0.006 ± 0.001, $n = 3$) (Fig. 4D).

4. Discussion

The importance of IRBIT remains incompletely understood. Relatively high IRBIT expression levels are found in epithelial tissues such as testis, ovaries, lung, kidney, and spleen [1]. The most well-known function of IRBIT is regulation of the activities of the Na^+-HCO_3^- co-transporter, NBCe1-B [4,19] and the cystic fibrosis transmembrane conductance regulator CFTR [5].

Several of our findings suggest that NHE3 participates in pH_i regulation in salivary epithelial cells, and IRBIT plays a vital role in pH_i regulation via its effect on NHE3 translocation from cytosol to plasma membrane in response to cell acidification. First, NHE3 interacts with IRBIT, and not only expression levels of NHE3, but also the amount of NHE3-IRBIT complex at the membrane were significantly increased by cell acidification. Second, even in the presence of high concentrations of HOE642, an NHE1-specific

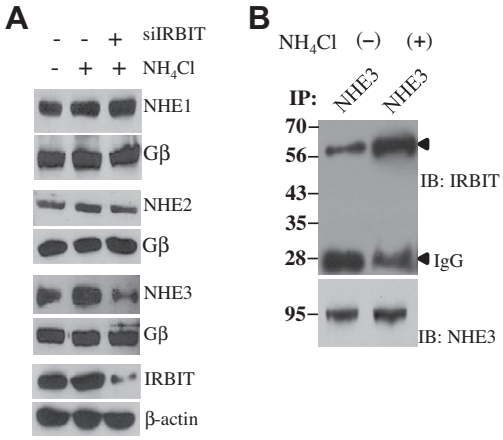

Fig. 3. The effect of IRBIT on the translocation of NHE3. (A) Western blot analysis with an anti-NHE1, anti-NHE2, anti-NHE3, and anti-G_β antibodies at the plasma membrane. Whole-cell lysates were subjected to western blotting with anti-IRBIT and anti-β-actin antibodies. By NH_4Cl pulse, membrane expression levels of all NHE subtypes, 1, 2 and 3, are increased by ~54%, ~16%, and ~49%, respectively (2nd lanes in Fig. 3A) compared to the controls (1st lanes in Fig. 3A). Silencing of endogenous IRBIT did not affect the membrane expression levels of NHE1 and 2, but significantly reduced the amount of membrane-localized NHE3 by ~34% (3rd lane in Fig. 3A). (B) IP with anti-NHE3 antibody in two groups: control (1st lane) and following a NH_4Cl pulse (2nd lane). The amount of IRBIT co-precipitating with NHE3 was significantly increased by ~49% comparing to the control ($n = 3$).

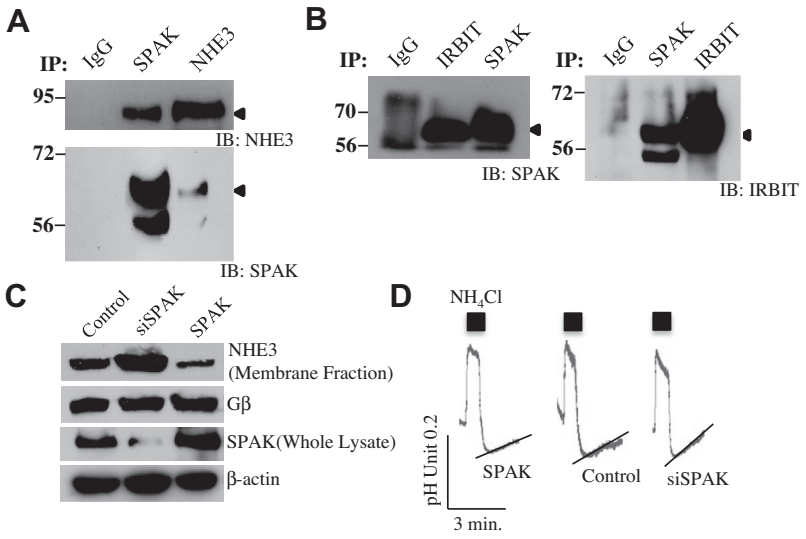

Fig. 4. The inhibitory effect of SPAK on IRBIT-induced NHE3 translocation. (A) IP with mouse IgG, anti-SPAK and anti-NHE3 antibodies. IPs were subjected to western blotting with anti-SPAK ($n = 2$) and anti-NHE3 ($n = 3$) antibodies. (B) IP with mouse IgG, anti-IRBIT and anti-SPAK antibodies. IPs were subjected to western blotting with anti-SPAK ($n = 2$) and anti-IRBIT ($n = 2$) antibodies. (C) Transfection with a siRNA sequence targeting SPAK in 10 nM and 10 μl SPAK. Western blot analysis with an anti-NHE3 and anti-Gβ antibodies at the plasma membrane. Whole cell lysate was subjected to western blotting with anti-SPAK and anti-β-actin antibodies. (D) Na^+-dependent recovery after NH_4Cl pulse (black bars) in HSG cells transfected with siSPAK, SPAK, or scrambled siRNA (control, the middle figure) in a HCO_3^--free solution. Silencing of endogenous SPAK enhanced pH_i recovery by ~16% (the right figure), while overexpression of SPAK inhibited pH_i recovery by ~18% (the left figure).

inhibitor, ~40% of pH_i recovery was still observed, possibly mediated by NHE2 and/or NHE3. HCO_3^--dependent pH_i recovery, for example, mediated by NBCe1B, could be excluded, since there was no HCO_3^- in our bath solution. Third, silencing of siIRBIT, which interacts only with NHE3, significantly reduced pH_i recovery from the cell acidification. Therefore, our results strongly suggest that IRBIT plays an important role in pH_i regulation in HSG cells, which were originally cultured from human submandibular ducts. Unfortunately, we cannot directly measure the pH_i recovery mediated by NHE3, since an NHE3-specific inhibitor is not currently commercially available.

It is not clear at the moment why IRBIT interacts only with NHE3. Although the amino acid sequences of the transport domains are highly homologous among the NHE subtypes, there is much greater divergence among the NHEs in the C-terminus [20]. The large, hydrophilic cytosolic domain of NHE1 (amino acids 500–815), which regulates the activity of the integral membrane domain, is the target for phosphorylation by protein kinases and for the binding of regulatory proteins [21]. Several protein kinases are thought to phosphorylate NHE1, including Ca^{2+}/calmodulin, Erk1/2, p90rsk, p38, and Nck-interacting kinase [22,23], while NHE3 activity has been found to be acutely regulated by several cytosolic proteins, including NHERFs 1, 2, 3, 4, CK2, phosphatidylinositol 3-kinase (PI3-K), CaM KII, and PLCγ [20,23]. Results from previous studies, showing binding of IRBIT to the Na^+/H^+ exchanger NHE3 and regulation of NHE3 activity by IRBIT [24,25], support our result.

IRBIT has multiple phosphorylation sites in the PEST domain, which is essential for the interaction with NHE3 [25]. The specific region between amino acids 591–696 of NHE3, which is highly hydrophilic, is necessary for the interaction with IRBIT and a previous study showed that the Ca^{2+}/CaM/CaMKII signaling axis regulates IRBIT-dependent trafficking of NHE3 [25]. Our data have shown that membrane NHE3 abundance was considerably enhanced and that the binding affinity of IRBIT to NHE3 was significantly increased in response to cell acidification. Thus our result strongly suggests that IRBIT enhances NHE3 translocation from cytosolic vesicles to plasma membrane during cell acidification.

SPAK is endogenously expressed in HSG cells. We obtained results similar to previous studies regarding the regulatory role of SPAK on NBCe1B [4,5,16]. SPAK also phosphorylates and activates other ion co-transporters including NCC, NKCC1, and NKCC2 [26,27]. In this study, we found that SPAK binds to IRBIT and NHE3 and regulates NHE3 activity. Similar to the SPAK effects on NBCe1-B activity, our data have shown that SPAK reversed the effect of IRBIT on NHE3 activity; knock down of SPAK increased IRBIT-induced membrane NHE3 expression, whereas SPAK overexpression decreased IRBIT-induced membrane NHE3 expression. In summary, we found that IRBIT, mediated by NHE3, which is further regulated by SPAK, plays an important role in pH_i recovery following cell acidification in salivary epithelial cells.

Acknowledgments

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