

The lateral mobility of NHE3 on the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP

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Summary

The epithelial brush border (BB) Na⁺/H⁺ exchanger, NHE3, plays a major role in transcellular Na⁺ absorption in the renal proximal tubule. NHE3 activity is rapidly regulated by neurohumoral substances and growth factors via changes in its amount on the BB by a process partially involving vesicle trafficking. The PDZ domain-containing proteins, NHERF1/2, are scaffold proteins that link NHE3 to the actin cytoskeleton via their binding to both ezrin and NHE3. NHERF1/2 interact with both an internal C-terminal domain of NHE3 and the N-terminus of ezrin. We used fluorescence recovery after photobleaching (FRAP) to study the effect of NHERF1/2 on NHE3 mobility in the brush border of opossum kidney (OK) proximal tubule cells. A confocal microscope was used to allow the selective study of apical membrane versus intracellular NHE3. A chimera of NHE3-EGFP was transiently expressed in OK cells and its lateral diffusion in the apical membrane was measured with FRAP and confocal microscopy at 37°C. The contribution of intracellular NHE3-EGFP to recovery on the OK surface not directly over the juxtannuclear area (non-JN) was negligible as exposure to the water soluble crosslinker BS³ (10 mM) at 4°C resulted in no recovery of this component of surface NHE3-EGFP after photobleaching. The mobile fraction (M_f) of apical NHE3-

EGFP was 47.5±2.2%; the effective diffusion coefficient (D_{eff}) was (2.2±0.3)×10⁻¹⁰ cm²/second. Overexpression of NHERF2 in OK cells decreased the M_f to 29.1±3.1% without changing D_{eff}. In the truncation mutant, NHE3585-EGFP (aa 1-585), which lacks the NHERF1/2 binding domain, M_f increased to 66.4±2.2%, with no change in D_{eff}, whereas NHE3660-EGFP, which binds NHERF1/2, had M_f (48.3±3.0%) and D_{eff} both similar to full-length NHE3. These results are consistent with the PDZ domain proteins NHERF1 and NHERF2 scaffolding NHE3 in macromolecular complexes in the apical membrane of OK cells under basal conditions, which limits the lateral mobility of NHE3. It is probable that this is one of the mechanisms by which NHERF1/2 affects rapid regulation of NHE3 by growth factors and neurohumoral mediators. By contrast, disrupting the actin cytoskeleton by latrunculin B treatment (0.05 μM, 30 minutes) reduced the NHE3 M_f (21.9±4.5%) without altering the D_{eff}. Therefore the actin cytoskeleton, independently of NHERF1/2 binding, is necessary for apical membrane mobility of NHE3.

Key words: Na⁺/H⁺ exchange, Brush Border, NHE3, Na⁺ absorption, FRAP

Introduction

The brush border (BB) Na⁺/H⁺ exchanger, NHE3, plays a major role in transcellular Na⁺ transport in epithelial cells of the small intestine, colon, gallbladder and renal proximal tubule (Hoogerwerf et al., 1996; Silviani et al., 1997). NHE3 has been recognized molecularly since 1992 and, as all mammalian NHEs, is made up of two domains, a relatively conserved ~450 aa N-terminal transport domain and a divergent ~400 aa C-terminal regulatory domain. Nearly all rapid regulation of NHE3 activity requires the intracellular C-terminus, aa 455-832. The NHE3 C-terminus acts as a scaffold, binding multiple proteins and lipids involved in either basal or stimulated regulation of NHE3. These include phosphatidylinositol

(4,5)-bisphosphate, which binds the C-terminus. Calcineurin homologous protein (CHP), calmodulin, calmodulin kinase II, megalin and protein phosphatase 2A (PP2A), as well as the two PDZ domain-containing proteins NHERF1 and 2, also bind to the C-terminus of NHE3 (Aharonovitz et al., 2000; Pang et al., 2001; Silva et al., 1995; Biemesderfer et al., 1999; Yun et al., 1998; Lamprecht et al., 1998; Zizak et al., 2003).

Because of the relevance of NHE3 to digestive and renal physiology and to the pathophysiology of diarrhoeal and renal diseases, multiple studies of the mechanisms of rapid regulation of NHE3 have been reported. Under basal conditions, NHE3 exists in the BB and intracellularly in a compartment that colocalizes with markers of the recycling

endosomes, including cellubrevin. NHE3 traffics between these two regions under basal conditions (D'Souza et al., 1998; Akhter et al., 2002). Most rapid regulation of NHE3 appears to involve changes in the amount of BB NHE3, which occurs from changes in the rates of NHE3 trafficking between the BB and the intracellular recycling compartment (Kurashima et al., 1998). In addition, changes in turnover number of NHE3, separate from changes in trafficking, also contribute (Cavet et al., 1999; Cavet et al., 2001). Moreover, rather than existing as isolated molecules in the plasma membrane, NHE3 exists as part of large plasma membrane complexes (Li et al., 2001). Some of the NHE3-associating complexes change in size as part of regulation, suggesting that rapid regulation of NHE3 is accompanied by changes in the nature and/or number of associating proteins.

PDZ domain-containing proteins are involved in protein-protein interactions and are now recognized as important organizers of protein complexes, predominantly at the plasma membranes, including in the BB of epithelial cells and in neural synapses. In epithelial cells, PDZ domain-containing proteins tend to localize in distinct membrane domains such as BB, rather than being present diffusely, and in most cases they colocalize with their binding partners. PDZ domain proteins have been shown to have multiple functions, many of which are exerted on plasma membrane binding partners. These include increasing trafficking, affecting both or either regulated endocytosis and exocytosis; anchoring in the plasma membrane, thus increasing plasma membrane retention; clustering; forming complexes; and affecting regulation by signal transduction including allowing changes in phosphorylation (for reviews, see Harris and Lim, 2001; Fanning and Anderson, 1999; Hung and Sheng, 2002; Sheng and Sala, 2001). In addition, PDZ domain proteins have been shown to be involved in the development of epithelial cell polarity (Bilder et al., 2003).

Studies of the role of NHERF1/2 in NHE3 location and regulation have been reported but have not been comprehensively evaluated in intestinal or renal epithelial cells. Most PDZ domain binding proteins interact with PDZ domains via their C-terminal 3-5 aa. However, some PDZ domain binding proteins also recognize internal peptide motifs. The best characterized example of an internal-motif-mediated PDZ interaction is the heterodimer involving the PDZ domain of nNOS and the PDZ domain of either syntrophin or PSD95 (Christopherson et al., 1999). NHE3 also binds to NHERF1 and 2 via an internal PDZ domain binding motif, which is between aa 589-660, based on *in vitro* interactions (Yun et al., 1998). In PS120 fibroblasts, neither the presence of NHERF1 nor NHERF2 altered the percent of NHE3 on the plasma membrane. This suggested that NHERF1 and NHERF2 were not involved in targeting NHE3 to the plasma membrane in fibroblasts (M. Cavet, B.C. and M.D., unpublished). In fibroblasts and the polarized renal proximal tubule opossum kidney (OK) cell line, cAMP inhibition of NHE3 requires the presence of either NHERF1 or NHERF2 (Yun et al., 1997). These PDZ proteins appear to be equivalent for cAMP regulation, which involves a complex of NHE3, NHERF1 or 2, ezrin, F-actin and protein kinase A II, with ezrin appearing to act as a low-affinity A kinase anchoring protein (AKAP) allowing cAMP to phosphorylate NHE3 (Zizak et al., 2000; Yun et al., 1998). Although there is no evidence that the

NHE3/PDZ domain-containing complex changes with cAMP regulation, cAMP does decrease the amount of NHE3 on the BB of OK cells (Szaszi et al., 2001). Elevated Ca^{2+} inhibition of NHE3 is also dependent on a PDZ domain protein, but unlike cAMP regulation, only NHERF2 and not NHERF1 can reconstitute Ca^{2+} inhibition of NHE3. The complex involved in Ca^{2+} regulation includes NHE3, NHERF2, α -actinin-4 and PKC α , with actinin-4 and PKC being necessary for Ca^{2+} regulation of NHE3. The reason that NHERF2 but not NHERF1 allows this Ca^{2+} regulation is that NHERF2 but not NHERF1 binds actinin-4 into the NHE3 complex (Kim et al., 2002).

The current studies have begun to evaluate the role of NHERF1 and 2 in NHE3 localization and mobility in the BB of living OK cells. Although the description of NHE3 as part of large complexes has been accomplished biochemically and via molecular manipulations, the consequences of these associations on lateral mobility of NHE3 in the plasma membrane has not been defined. FRAP was used to evaluate lateral mobility of NHE3 in the BB and in the recycling compartment and to determine the effect on lateral mobility of NHE3 association with NHERF1 and 2.

Materials and Methods

Materials

DSS (disuccinimidyl suberate), a membrane-soluble cross-linking reagent and BS³ [Bis(sulfosuccinimidyl) suberate], a water-soluble cross-linking reagent were from Pierce. LY294002 and anti-ezrin mAb were from Sigma. Latrunculin B was from Alexis Biochemicals. Cytochalasin D was from A.G. Scientific. Jasplakinolide, wheat germ agglutinin Alexa Fluor 350 conjugate and Alexa Fluor 568 phalloidin were from Molecular Probes.

Molecularly engineered constructs

Full-length and truncated NHE3-EGFP fusion proteins were assembled using the pEGFP-N3 vector (Clontech, CA). Wild-type full-length rabbit NHE3 cDNA was used as a template for all polymerase chain reactions (PCRs) described below. PCR was performed with primers to introduce *NheI* and *XhoI* restriction sites into the 5' and 3' ends, respectively, of the full-length and truncated NHE3 cDNAs. Wild-type NHE3 (aa 1-832), NHE3585 (aa 1-585) and NHE3660 (aa1-660) cDNAs were ligated into the pEGFP-N3 vector (Clontech, Palo Alto, CA) in frame with the C-terminal EGFP coding sequence, creating NHE3-EGFP, NHE3585-EGFP and NHE3660-EGFP, respectively. All constructs were fully sequenced before study. We previously showed that NHE3-EGFP localized in OK cells similarly to wild-type, being distributed in the apical membrane and in the juxtannuclear area. It was functionally inhibited by phosphoinositide 3-kinase inhibition (wortmannin), similarly to wild-type studies (Akhter et al., 2002). We have also reported that NHE3-EGFP stably expressed in PS120 cells behaves similarly to wild-type in terms of the percent amount on the surface, basal transport and stimulation by growth factors (Janecki et al., 2000a). Rab 11a was engineered to contain GFP on its N-terminus. The plasmid containing EGFP-Rab 11a (pEGFP-C2, Clontech) was kindly provided by J. Goldenring, Vanderbilt University School of Medicine. The plasmid containing YFP-GL-GPI (pEGFP-N1, Clontech) was kindly provided by K. Simons (Keller et al., 2001).

Cell culture and transfection

OK cells were cultured on glass-bottom 35 mm plastic culture dishes in DMEM-media (w/o phenol red), supplemented with 10% fetal

bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂/95% air atmosphere. The intrinsic NHE3 activity in OK cells was markedly decreased by repeated applications (one per week) of the 'Acid suicide' technique of Pouyssegur (Pouyssegur et al., 1984). OK cells were incubated with 130 mM LiCl (pH_o, 7.4) for 2 hours. Then the LiCl solution was removed and incubated with Na⁺, Li⁺-free choline Cl (130 mM, pH 5.5) for 60 minutes. This resulted in OK cells with low NHE3 activity (Fig. 1), such that the NHE3 present after transient transfection with NHE3-EGFP or NHE3585-EGFP was nearly all the transfected chimera.

OK cells with low BB NHE3 activity were then transfected with 2 µg of EGFP constructs at ~100% confluency using 10 µl of Lipofectamine 2000 (Invitrogen) in 35-mm glass-bottom Petri dishes using the manufacturer's instructions. Cells then were grown in serum free D-MEM/F-12 media without phenol red (Life Technology) with 600 units/ml G418 (GibcoBRL) for 48 hours before study. D-MEM/F-12 w/o phenol red media was used to decrease the background fluorescence.

Immunofluorescence

OK cells were studied for immunofluorescence 48 hours after transient transfection with NHE3-EGFP constructs using the above conditions. Cells were fixed with 3% formaldehyde in phosphate buffered saline (PBS), neutralized with 20 mM glycine in PBS for 10 minutes at room temperature and permeabilized with PBS containing 0.1% saponin for 20 minutes. Cells were then blocked for 30 minutes in 1% BSA/PBS supplemented with 10% FBS. Cells were first incubated with primary antibodies for 60 minutes with polyclonal antibody 2570 (anti-NHERF2 antibody) or monoclonal anti-ezrin antibody (Sigma). After three 10 minute washes in 0.1% saponin/PBS, secondary antibodies (Alex Fluor 546-anti mouse for Ezrin or Alex Fluor 568-anti rabbit for NHERF2; Molecular Probes) were added in PBS, incubated for 30 minutes and again washed three times for 10 minutes before images were taken. Actin filaments were stained with Alexa Fluor 568 Phalloidin (Molecular Probes). Cells were examined with a confocal fluorescence microscope (Zeiss LSM 410).

Photobleaching experiments

FRAP (for a review, see Edidin, 1994) was performed on a stage heated to 37°C of a Zeiss LSM 410 confocal microscopy using the 488 nm line of a 400-mW Kr/Ar laser in conjunction with a 100× Zeiss 1.4 NA Planapochromat oil immersion objective (high NA lens). To maintain temperature, a DH-35, 35 mm Culture Dish Heater was used. A homemade adapter was used for mounting the DH-35 on the Zeiss LS 410 microscope stage. The heater was used with the Warner Instrument heater controller TC-324B. The cable assembly included the two heater connectors, control thermistor and an additional thermistor to measure temperature in the dish.

Time lapse imaging was performed using 'macros' programmed with the Zeiss LSM software package that allowed autofocusing on the coverslip surface in the reflection mode before taking confocal fluorescence images. The scanning laser intensity did not significantly photobleach the specimen over the time course of the experiment. For quantitative measurements of mobile fraction and diffusion coefficient, a photobleached rectangle 4 µm wide was used that extended across the cell and through its entire depth. Fluorescence within the strip was measured at low laser power (20% power, 1% transmission) before the bleach (pre-bleach intensity) and then photobleached with full laser power (100% power, 100% transmission). Recovery was followed with low laser power at 9-second intervals usually up to 50 images until the intensity had reached a steady plateau. To D_{eff}, the experimental data was fit to an empirical formula given in equation (1) (Ellenberg et al., 1997):

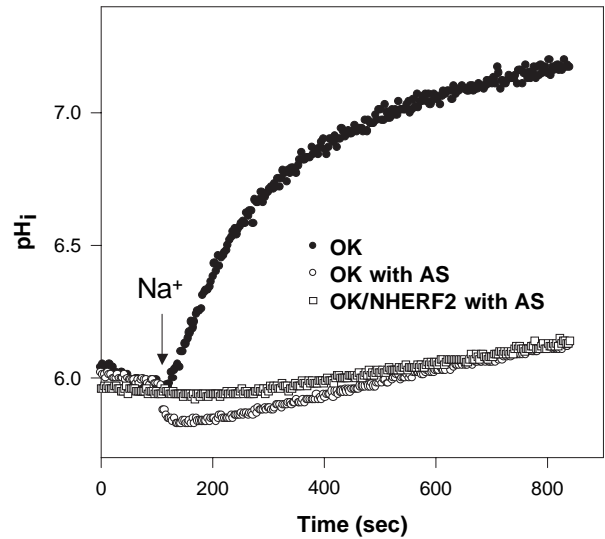


Fig. 1. OK and OK/NHERF2 cells were selected by acid suicide to be NHE3 null. OK cells, which had been cloned to remove fibroblast contamination (Noel et al., 1996), were selected by acid suicide (see Materials and Methods). The intracellular pHi was measured with BCECF over time, following acidification by NH₄Cl pulsing (Levine et al., 1993). Arrow indicates Na⁺ addition. AS refers to acid suicide. NHE3 null OK cells were used for transient transfection.

$$F(t) = F_0 + F_\infty \{1 - [w^2(w^2 + 4\pi D_{\text{eff}}t)^{-1}]^{1/2}\} \quad (1)$$

with F(t)=intensity as a function of time; F₀=intensity just after bleaching; F_∞= final intensity reached after complete recovery; w=strip width, i.e. 4 µm; D_{eff}=effective one-dimensional diffusion constant. This equation was used on the assumption that the unbleached material around the strip has the same fraction of mobile/immobile molecules as within the strip, and that only the mobile molecules can recover. Effective diffusion constants were calculated by fitting this function to the experimental data. The mobile fraction was determined by comparing the fluorescence intensity in the bleached region after full recovery (F_∞) with the fluorescence intensity before bleaching (F_i) and just after bleaching (F₀). Mobile and immobile fractions were calculated by comparing the intensity ratio in regions of interest inside and outside the bleached area just before the bleach and after recovery as shown in equation (2).

$$M_f = [(F_\infty - F_0)/(F_i - F_0)] \times 100(\%) \quad (2)$$

The postbleach intensities were normalized to correct for maximal loss of fluorescence due to the photobleach. Fluorescence intensity was normalized to prebleach intensity (F_i) corrected for maximal loss of fluorescence. The intensity measurements of the bleached box as a time course were performed using MetaMorph software (Universal Imaging Corp.). The intensity data were converted to text files using Excel software for further analysis. Curve fitting analysis, using Eq. (1) and (2), was performed with Origin 6.0 (Microcal) software to evaluate mobile fraction and effective diffusion coefficient. All data are shown as mean±s.e.m. (standard error of the means) of the number of cells analyzed, which were obtained in at least three identical experiments, unless stated otherwise. Statistical comparison was performed by unpaired Student's *t*-test.

Results

NHE3-EGFP localizes in the juxtannuclear region and apical surface

The mobility of NHE3 in living cells was determined using C-

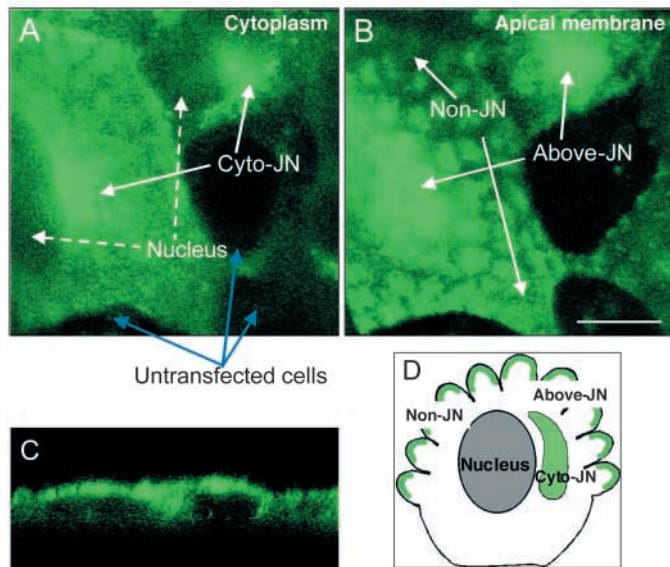


Fig. 2. NHE3-EGFP expression in transiently transfected OK cells. Images were collected ~48 hours after transfection with NHE3-EGFP, during which time the cells were incubated with serum-free media. Fluorescence micrographs were taken with a 100 \times objective. NHE3-EGFP localized in three pools in OK cells: in the juxtannuclear (JN) area (as previously described) (D'Souza et al., 1998; Akhter et al., 2002) (A), in the microvilli of the apical plasma membrane above the JN or in a non-JN area (B). Bar, 10 μ m. (C) XZ reconstruction showing NHE3-EGFP distribution in apical membrane and JN area. (D) The three pools of NHE3-EGFP in OK cells. A and B were oversaturated on purpose to show both microvillus and intracellular NHE3 locations.

terminally tagged EGFP as a fluorescent reporter. Transient transfection of OK cells with wild-type NHE3-EGFP using the protocol described when studied 48 hours after transfection was ~20% efficient. NHE3-EGFP localized in three different regions in OK cells (Fig. 2): cytoplasmic juxtannuclear (cyto-JN) region, apical plasma membrane above-JN and apical plasma

membrane non-JN. Apical surface expression of NHE3-EGFP was on microvilli with much less in the intervillus areas, as reported previously (Hu et al., 2001). The juxtannuclear domain localization of NHE3 was previously shown to consist largely of the recycling endosomes in OK and PS120 cells (D'Souza et al., 1998; Janecki et al., 2000a; Akhter et al., 2002). FRAP was analyzed on these three different regions of NHE3-EGFP. The mobile fractions and effective diffusion coefficients were similar in the three regions, being (1) $M_f=44.0\pm 3.1\%$, $D_{eff}=(2.9\pm 0.3)\times 10^{-10}$ cm²/second for cyto-JN, (2) $M_f=46.2\pm 2.2\%$, $D_{eff}=(2.3\pm 0.3)\times 10^{-10}$ cm²/second for apical non-JN, and (3) $M_f=47.8\pm 2.7\%$, $D_{eff}=(2.1\pm 0.2)\times 10^{-10}$ cm²/second for apical above-JN (Fig. 3). These parameters for fluorescence recovery were similar to other plasma membrane proteins, including Na/K-ATPase (Paller, 1994), EGF receptor (Livneh et al., 1986), aquaporin-1 (Cho et al., 1999) and the integrin alpha IIIb beta3 (Schootemeijer et al., 1997).

As further evidence that it was apical membrane NHE3-EGFP that was being studied, rather than intracellular NHE3, it was determined whether a marker of the apical recycling endosome appeared in the confocal microscopy window (XY section) used for the FRAP studies. EGFP-Rab 11a, as previously engineered (J. Goldenring, personal communication), was transiently transfected into OK cells. Cells that gave bright signals for EGFP-Rab 11a were examined. There was no fluorescence in the XY sections used for studies of apical membrane NHE3 lateral mobility as marked with wheat germ agglutinin at 4 $^{\circ}$ C (data not shown).

The internal contribution to the fluorescence recovery of NHE3-EGFP in the apical membrane non-JN region was negligible but was significant in the apical membrane above-JN region

Because the plasma membrane half-life of NHE3 was ~15 minutes when expressed in fibroblasts and in OK cells (Kurashima et al., 1998; Cavet et al., 2001) (M.D., unpublished) and the time it took for fluorescence recovery to approach steady state in these studies was ~5 minutes, partial

Fig. 3. FRAP analysis of NHE3-EGFP in the three regions in OK cells. Fluorescence photobleaching was performed and recovery analyzed in the three regions shown in Fig. 2 – intracellular JN, apical surface above-JN and non-JN of NHE3-EGFP in OK cells. Images show prebleaching (A), immediately post bleaching (B) and after recovery (450 seconds after bleaching) (C) at the level of the apical plasma membrane. The prebleaching intensity (F_i), intensity just after bleaching (F_0) and final intensity after full recovery (F_{∞}) are shown in D. FRAP data were collected every 9 seconds up to 50 images. The mobile fraction (M_f) and effective diffusion coefficient (D_{eff}) of NHE3-EGFP in the three pools in OK cells were obtained by fitting the photobleaching curve according to Materials and Methods (see E and F). $n=12$ cells studied for Cyto-JN, $n=15$ for above-JN and $n=21$ for non-JN. Data were from three separate experiments. P values for M_f were in comparison to cyto-JN (unpaired t -test).

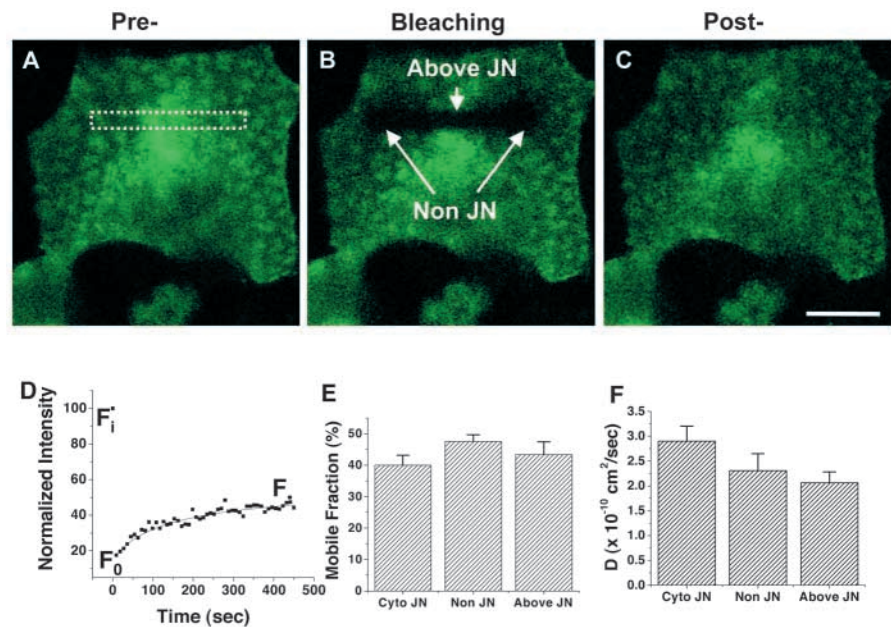
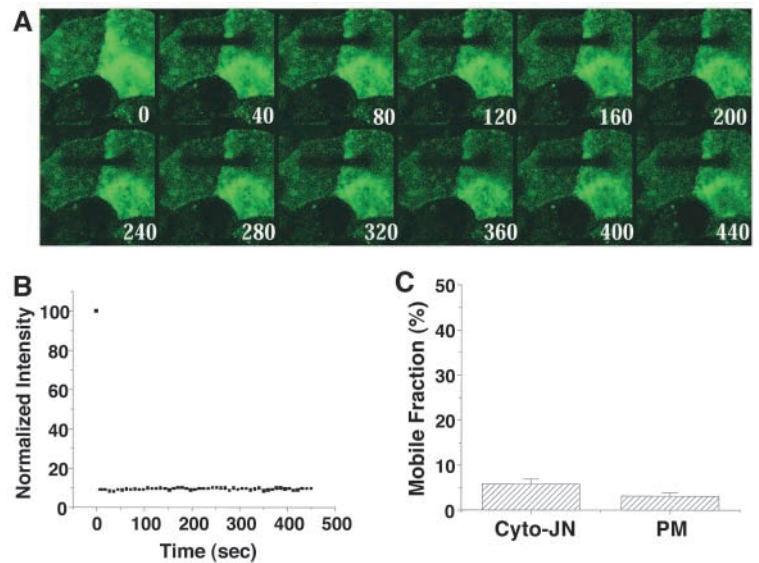


Fig. 4. NHE3-EGFP did not recover after photobleaching in either the apical plasma membrane or JN areas after DSS cross-linking. OK/NHE3-EGFP cells had fluorescence recovery studied after the cells were exposed to the membrane-penetrable cross-linking reagent DSS (disuccimidyl suberate) (1 mM for 1 hour at 37°C). The NHE3-EGFP fluorescence did not recover in either the apical domain or in the intracellular juxtannuclear (JN) region after photobleaching. (A) Time-course montage after photobleaching (the numbers at the bottom are seconds after the bleach) of apical membrane NHE3-EGFP in OK cells. Magnification 100× objective. (B) Quantitative fluorescence recovery of NHE3-EGFP in the apical plasma membrane. Similar results were obtained of NHE3-EGFP in the intracellular JN domain. Note, the apical membrane studied (A and B) included NHE3-EGFP over the JN plus the non-JN domain. Mobile fractions for NHE3-EGFP in Cyto-JN and the apical plasma membrane (PM) are shown in C. $n=5$ for Cyto-JN and $n=6$ for PM from two separate experiments.



recovery of the mobile fraction might have been due to trafficking to the apical membrane. Thus, studies were performed to assess the contribution of trafficking of intracellular NHE3-EGFP to the recovery of apical surface NHE3 after photobleaching. The lipid soluble cross-linking reagent DSS (disuccimidyl suberate) was exposed to OK/NHE3-EGFP cells (1 mM, 1 hour). The M_f of NHE3-EGFP with DSS treatment at the intracellular juxtannuclear region and at the apical plasma membrane (entire apical NHE3-EGFP analyzed) was $5.8 \pm 1.1\%$ and $3.1 \pm 0.7\%$, respectively. That is, DSS almost completely blocked the mobile fraction of NHE3-EGFP both at the apical plasma membrane (no difference between above-JN and non-JN) and juxtannuclear regions (Fig. 4). Thus, in the presence of lipid soluble cross-linker, the mobile fractions of all regions of NHE3 were reduced to almost zero.

To assess the contribution of intracellular NHE3 to fluorescence recovery of apical membrane NHE3, similar studies were repeated with the water-soluble cross-linker BS³ at 4°C. Unlike DSS, which crosslinks both intracellular and cell-surface NHE3, BS³ can only access NHE3 present on the apical plasma membrane. Therefore, BS³ can be used to distinguish recovery caused by trafficking from the intracellular NHE3 pool versus lateral diffusion in the apical membrane. OK/NHE3-EGFP cells were incubated with BS³ (10 mM) at 4°C for 30 minutes and then quickly moved to the 37°C stage to measure fluorescence recovery. Fluorescence recovery studies were performed of the three regions of NHE3-EGFP in OK cells (Fig. 5). The mobility of NHE3-EGFP at the cytoplasmic JN region was not affected by BS³. M_f was $45.0 \pm 4.8\%$ (similar to control, see Fig. 3). By contrast, BS³ almost completely blocked the apical non-JN NHE3-EGFP recovery; M_f was dramatically reduced to $6.3 \pm 1.8\%$. Although BS³ almost completely blocked NHE3-EGFP M_f in the apical non-JN pool, there was still a significant mobile fraction of NHE3 in the apical-JN region in the presence of BS³ ($M_f = 22.6 \pm 4.0\%$), with only a partial decrease of the M_f .

The differences in the mobile fractions in these two apical membrane regions of NHE3 in the presence of BS³ indicates that trafficking contributes to fluorescence recovery of apical

membrane NHE3 in the region above the intracellular JN domain but not that away from the JN region. This strongly suggests that trafficking between the recycling compartment and the apical membrane does not occur uniformly and rather predominantly occurs in a localized region above the cytoplasmic JN region. In order to study lateral mobility of NHE3 in the apical membrane and not a combination of apical membrane mobility and trafficking, the rest of the studies concentrated on fluorescence recovery in the apical non-JN region.

Inhibition of phosphoinositide 3-kinase with LY294002 did not significantly change fluorescence recovery of NHE3-EGFP in OK cells in spite of decreasing apical NHE3 by 50%

NHE3 stably transfected in PS120 and AP-1 fibroblasts and in OK cells rapidly traffics between the recycling endosomes and the cell surface in a phosphoinositide 3-kinase-dependent manner under 'basal conditions'. In fibroblasts and OK cells, wortmannin, a potent inhibitor of phosphoinositide 3-kinase, reduced the basal transport rate and surface levels of NHE3 by ~50%, which in fibroblasts was shown to be due to inhibition of the exocytosis of the exchanger back to the plasma membrane (Kurashima et al., 1998; Janecki et al., 2000b; Akhter et al., 2002). Further studies were performed to confirm that fluorescence recovery in non-JN apical NHE3 in OK cells was not influenced by decreasing NHE3 trafficking and to compare the fluorescence recovery of the trafficking NHE3 to that of the total. The effect of LY294002, another phosphoinositide 3-kinase inhibitor, was examined on apical membrane NHE3-EGFP in OK cells. LY294002 (50 μ M, 30 minutes) reduced apical NHE3 activity by ~50% (data not shown). Nonetheless, the mobility and effective diffusion coefficient of NHE3-EGFP on the apical membrane away from the juxtannuclear compartments was not significantly changed by LY294002 treatment. The M_f was $50.5 \pm 4.5\%$ and D_{eff} was $(3.9 \pm 1.2) \times 10^{-10}$ cm²/second (Fig. 6). This result showed that the fluorescence recovery of the apical membrane NHE3-EGFP away from the JN region was not affected by inhibiting phosphoinositide 3-kinase activity and NHE3 exocytosis.

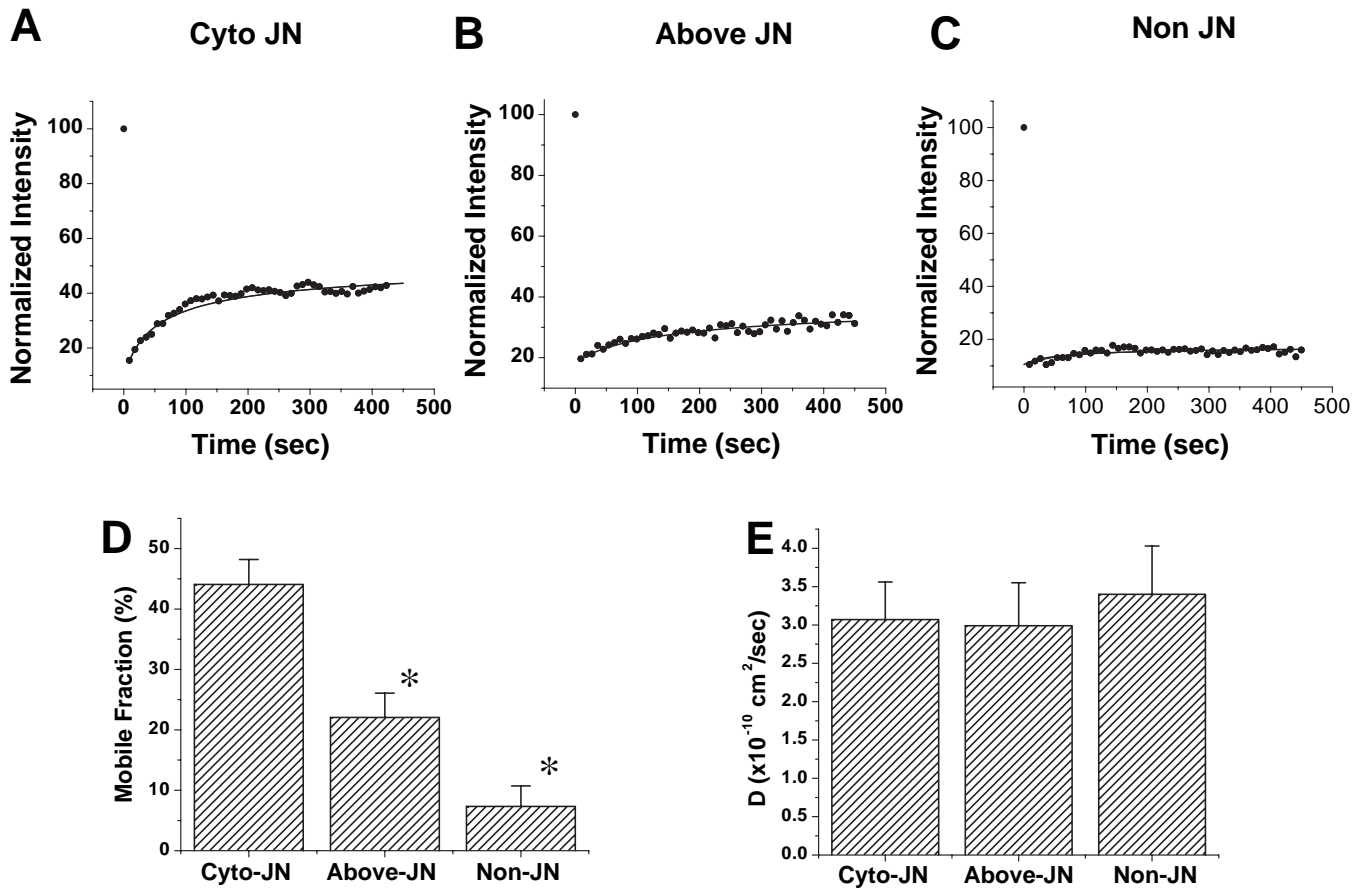


Fig. 5. The contribution to apical membrane NHE3-EGFP recovery after photobleaching of intracellular NHE3-EGFP was negligible when non-JN NHE3 was studied but was significant in the above-JN region. Fluorescence recovery was studied after 30 minutes exposure at 4°C to the water-soluble cross-linking reagent, BS³ (10 mM). NHE3-EGFP recovery was performed on the intracellular JN (cyto-JN) (A), apical plasma membrane above-JN (B) and non-JN (C) domains. The mobile fractions (D) and diffusion coefficients (E) of the NHE3-EGFP domains were determined by FRAP. Data shown are mean \pm s.e.m. **P* values compared with cyto-JN are shown (unpaired *t*-test). *n*=7 for cyto-JN and non-JN; *n*=6 for above-JN.

Moreover, it showed that NHE3 remaining after blocking exocytosis for 30 minutes had similar lateral mobility to the initial apical membrane pool of NHE3.

NHE3-EGFP colocalizes with NHERF2, ezrin and F-actin at the apical plasma membrane

Epithelial cells expressing ezrin generally co-express NHERF1. This is the case in intestinal epithelial cells, gastric parietal cells, the epithelial cells of the kidney proximal tubule and the terminal bronchioles of the lung (Ingraffea et al., 2002). In some epithelial cells, expression of NHERF1 and NHERF2 are mutually exclusive. This is not true in ileal BB, which has both NHERF1 and 2 (Li et al., 2001). Also, in the apical membrane of mouse and rabbit proximal tubules, there is both NHERF1 and 2 (E. Weinman and J. Wade, personal communication). OK cells endogenously express NHERF1 but not NHERF2 (Wade et al., 2001; Yun et al., 2002; Hernando et al., 2002). To study the role of NHERF1 and NHERF2 alone and together in NHE3 mobility (mimicking the situation in mouse/rabbit proximal tubule), we stably expressed NHERF2 in OK cells. OK cells with low NHE3 activity because of 'acid suicide' were used for NHERF2 stable transfections and also transiently transfected

with NHE3-EGFP. In OK cells expressing NHE3-EGFP and NHERF2, the latter colocalized with NHE3-EGFP on the apical membrane and in the cytosol (Fig. 7A). Ezrin indirectly binds to NHE3 via the PDZ domain proteins, NHERF1 or NHERF2. In OK cells, ezrin was also mostly expressed in the apical membrane in the microvilli and colocalized with NHE3-EGFP (Fig. 7B). The microvillus structures on the apical plasma membrane were confirmed by F-actin staining. F-actin distributes in OK cells in basal stress fibers (Fig. 7C1), the lateral domain (Fig. 7C2) and microvilli (Fig. 7C3) like in Caco-2 cells (Durrbach et al., 2000). Fig. 7D1-3 shows that NHE3-EGFP colocalized with F-actin at the apical plasma membrane microvilli area (see arrow a in Fig. 7D3), but the NHE3 present in the subapical juxtannuclear compartment did not colocalize with F-actin in this area (see arrow b in Fig. 7D3).

It was previously shown that NHE3 has an internal PDZ domain binding region at internal sequence aa 589-660 (Yun et al., 1998). OK/NHERF2/NHE3585-EGFP cells lack binding sites for NHERF1 and 2. In contrast to colocalization of NHERF2 with NHE3 in BB of OK/NHERF2/NHE3-EGFP cells (Fig. 7A), NHERF2 had a different distribution in OK/NHERF2/NHE3585-EGFP cells. In these cells, in spite of NHE3585 having an apical membrane pool (Fig. 7E1),

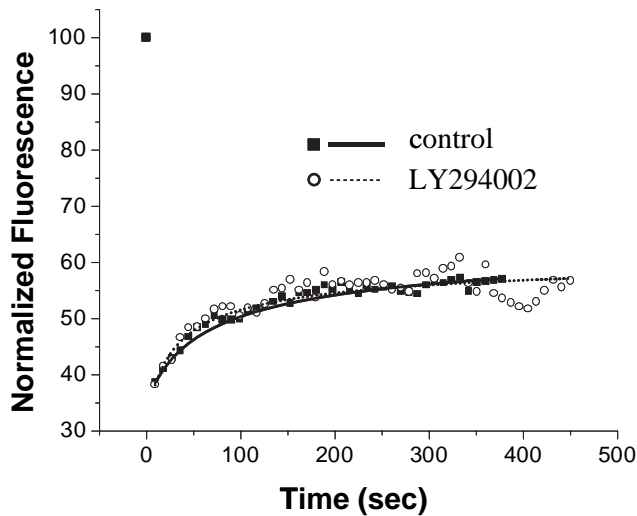


Fig. 6. Decreasing surface NHE3 by inhibiting phosphoinositide 3-kinase did not alter fluorescence recovery of non-JN NHE3-EGFP remaining in the apical surface. OK/NHE3-EGFP cells were incubated with 50 μ M LY294002 for 30 minutes at 37°C, then fluorescence recovery measurements were performed with analysis of apical non-JN NHE3-EGFP. The result shown is a representative FRAP experiment with initial fluorescence intensity before photobleaching set at 100%. Data were collected as 50 images every 9 seconds. $M_f=50.5\pm 4.6\%$ and $D_{eff}=(3.9\pm 1.2)\times 10^{-10}$ cm²/second, for six cells in two separate studies on both untreated and LY294002-treated cells. Data shown are mean \pm s.e.m.

NHERF2 was not localized to the apical membrane and rather was present diffusely throughout the cytosol (Fig. 7E2). However, NHERF1 had a normal apical membrane distribution and colocalized with NHE3585-EGFP in OK/NHE3585-EGFP cells (Fig. 7F).

NHERF1 or 2 limit lateral mobility of NHE3-EGFP in OK cells

To evaluate the role of the PDZ proteins NHERF1/2 on NHE3 mobility, OK cells stably transfected with NHERF2 and transiently transfected with NHE3-EGFP or NHE3585-EGFP were studied. NHERF2 colocalizes with NHE3-EGFP at the apical membrane in OK/NHERF2/NHE3-EGFP cells (see Fig. 7A). This is the same distribution reported for NHERF1 (Wade et al., 2001). Therefore, we hypothesized that stable expression of NHERF2 might enhance the effect of the endogenous NHERF1 on NHE3 mobility. Three cell lines were constructed, OK/NHE3-EGFP, OK/NHERF2/NHE3-EGFP and OK/NHERF2/NHE3585-EGFP. OK/NHE3-EGFP has intrinsic NHERF1 but lacks NHERF2; OK/NHERF2/NHE3-EGFP has intrinsic NHERF1 and stably expressed NHERF2; and OK/NHERF2/NHE3585-EGFP has both NHERF1 and NHERF2 but the NHE3585-EGFP construct has no PDZ binding domain for NHERF1/2. As shown in Fig. 8 in OK/NHE3-EGFP cells, M_f of apical non-JN wild-type NHE3-EGFP was $46.2\pm 2.2\%$ and D_{eff} was $(2.3\pm 0.3)\times 10^{-10}$ cm²/second. Stable expression of NHERF2 in OK/NHE3-EGFP cells significantly decreased the mobility to $29.1\pm 3.1\%$ ($P<0.01$) without changing D_{eff} ($= (2.2\pm 0.3)\times 10^{-10}$ cm²/second). The mobile fraction of the truncation mutant

NHE3585-EGFP, lacking the NHERF1 and NHERF2 binding domain, was increased to $66.4\pm 2.2\%$ without changing D_{eff} ($2.5\pm 0.3)\times 10^{-10}$ cm²/second). These results show that both NHERF1/2 may be involved in tethering NHE3 to the apical membrane and restrict its lateral mobility. We suspect that the effects of NHERF1 and NHERF2 are additive, given that when neither bound (NHE3585), M_f was highest, and when both NHERF1 and NHERF2 were expressed, M_f was lowest, whereas cells with only NHERF1 had an intermediate M_f . However, we have not provided definite evidence that NHERF1 alters basal M_f of NHE3.

Additional control studies were performed of OK/NHE3660-EGFP and OK/NHERF2/NHE3660-EGFP cells. These cells were studied because NHERF1/NHERF2 bind NHE3 between aa 585-660. We hypothesized that if differences in NHE3 mobility between wild-type and NHE3585 were due to a lack of NHERF1/2 binding, these differences would not be present in NHE3 truncated to aa 660. NHE3660-EGFP was present in microvilli of OK and OK/NHERF2 cells similar to wild-type NHE3-EGFP (data not shown). The mobile fraction and D_{eff} of NHE3660-EGFP (M_f , $48.3\pm 3.0\%$; D_{eff} , $2.6\pm 0.5)\times 10^{-10}$ cm²/second), were not different from full-length NHE3. Similarly, in NHE3660-EGFP/NHERF2 cells, the NHE3 M_f ($35.8\pm 3\%$) and D_{eff} ($3.2\pm 0.4)\times 10^{-10}$ cm²/second) were similar to full-length NHE3, and the M_f was reduced compared with OK/NHE3660 cells. These results support the role of NHERF1/NHERF2 in limiting the NHE3 M_f , although other proteins that might bind NHE3 in this domain could also be involved.

Disruption of actin filaments by latrunculin B equivalently inhibits the lateral mobility of apical membrane NHE3-EGFP and NHE3585-EGFP

The contribution of actin filaments to the lateral mobility of NHE3 on the microvilli was evaluated using latrunculin B, a drug that sequesters monomeric actin. After 30 minutes of incubation with latrunculin B (0.1 μ M) at 37°C, the actin filaments localized at the apical membrane microvilli structures became disorganized and microvilli were more patchy (Fig. 9A). In these cells, the amount of apical membrane NHE3-EGFP also was visibly decreased; after 30 minutes of latrunculin B exposure most of the NHE3-EGFP was intracellular (Fig. 9B). Conditions of latrunculin B exposure were found which only partially disrupted F-actin in the microvilli and kept NHE3-EGFP in the microvilli. With 0.05 μ M latrunculin B exposure, NHE3-EGFP was still present in microvilli after 30 minutes (Fig. 9C). Lateral mobility was assessed under these conditions on OK/NHE3-EGFP cells at the cytoplasmic JN and the apical non-JN domains. Latrunculin B (0.05 μ M, 30 minutes) treatment significantly decreased the mobile fraction of NHE3-EGFP to $M_f=11.2\pm 3.2\%$ (cf. $M_f=46.2\pm 2.2\%$; w/o latrunculin B) at the apical plasma membrane without changing D_{eff} [$(4.1\pm 1.8)\times 10^{-10}$ cm²/second] in OK/NHE3-EGFP cells (Fig. 9D, left and Fig. 9E, left, respectively). By contrast, neither the mobile fraction nor effective diffusion coefficient of the cytoplasmic JN NHE3-EGFP ($M_f=37.0\pm 8.3\%$ and $D_{eff}=(3.9\pm 1.3)\times 10^{-10}$ cm²/second) were changed with this latrunculin B treatment.

We also evaluated fluorescence recovery on OK/NHERF2/NHE3585-EGFP cells with the same conditions

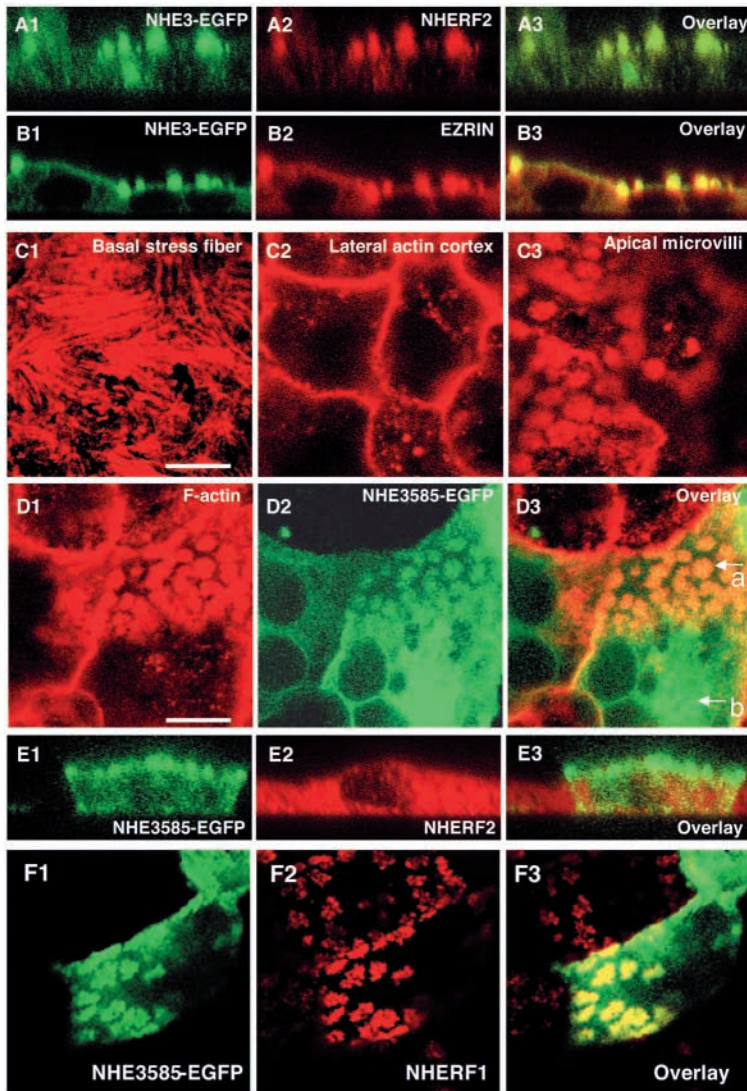


Fig. 7. NHE3-EGFP, NHERF2, ezrin and F-actin colocalize in the OK/NHERF2/NHE3-EGFP cell apical membrane. OK cells transiently expressing NHE3-EGFP were plated on a glass-bottom 35 mm plastic culture dish and processed for confocal imaging. Cells were fixed and labeled either with polyclonal anti-NHERF2 (A2 and E2), monoclonal anti-ezrin (B2) or phalloidin (C1-3 and D1). Secondary antibodies were either Alexa-568-conjugated goat anti-rabbit IgG for NHERF2 staining or Alexa-595-conjugated goat anti-mouse IgG for ezrin staining. The green channel shows the distribution of NHE3-EGFP and red channels show NHERF2 (A2, E2), ezrin (B2) and F-actin (C1-3, D1). Overlaid images are shown in the right column. XZ sections show colocalization of NHE3-EGFP with NHERF2 (A3), ezrin (B3) and F-actin (D3) in microvilli. C1-3 shows F-actin staining at the basal stress fibers (C1), the lateral cell surface (C2) and apical membrane microvilli (C3) in polarized OK cells. Images D1-D3 show F-actin (D1) and NHE3585-EGFP (D2) staining at the apical plasma membrane. D3 is a composite image between D1 and D2. F-actin colocalized with NHE3585-EGFP only at the microvilli (D3a) but not in the subapical juxta nuclear region (D3b). XZ section images E1-E3 show NHE3585-EGFP (E1) and NHERF2 (E2). E3 is a composite image between E1 and E2. NHERF2 did not colocalize with NHE3585-EGFP at the microvilli. However, NHERF1 still shows normal microvilli distribution at the microvilli (F2) and colocalized with microvillus NHE3585-EGFP (F3). These OK cells endogenously expressed ezrin and NHERF1, stably expressed NHERF2 and transiently expressed NHE3-EGFP. Images were taken with a 100 \times objective. Bars, 10 μ m.

of latrunculin B treatment (0.05 μ M, 30 minutes). M_f of apical non-JN NHE3585 significantly decreased to 21.9 \pm 4.5% (cf. M_f =66.4 \pm 2.3%; w/o latrunculin B), while D_{eff} was not changed (D_{eff} =(3.4 \pm 0.6) $\times 10^{-10}$ cm²/second) (Fig. 9D, right and Fig. 9E, right, respectively). The fact that latrunculin B had similar effects on mobility of full-length NHE3 and NHE3585 indicates that this cytoskeleton effect occurs independently of the NHERF1/NHERF2 association with NHE3.

Other actin filament-disrupting drugs, cytochalasin D and jasplakinolide, a cell-permeable macrocyclic peptide that inhibits F-actin turnover depolymerization and thus stabilizes actin (Gallo et al., 2002), were studied to further evaluate the role of the actin cytoskeleton in the lateral mobility of NHE3. Cytochalasin D treatment was standardized to leave some visible clumped microvilli (5 μ M, 20 minutes), and the fluorescence recovery on OK/NHERF2/NHE3585-EGFP cells was evaluated. M_f of apical non-JN NHE3585 significantly decreased to 36.6 \pm 5.6%, whereas D_{eff} was not changed (D_{eff} =(3.7 \pm 0.9) $\times 10^{-10}$ cm²/second). By contrast, jasplakinolide (1 μ M, 1 hour) did not significantly change the NHE3585-EGFP/NHERF2 M_f and D_{eff} (M_f =70.6 \pm 6.3% and D_{eff} =(1.8 \pm 0.3) $\times 10^{-10}$ cm²/second).

These results suggest that the actin cytoskeleton is important for the lateral mobility of NHE3-EGFP and NHE3585-EGFP on the apical plasma membrane in OK cells and that it has a similar effect in the presence and absence of NHERF1/2 binding to NHE3.

The actin cytoskeleton could be involved in affecting NHE3 mobility either via attachment to a myosin motor, as was recently suggested for NHE3 movement in renal proximal tubule cells (Biemesderfer et al., 2002), or actin filament assembly, as was recently shown to be required for endocytosis in yeast (Kaksonen et al., 2003). To attempt to separate these possibilities, ATP was depleted in the OK/NHERF2/NHE3 585-EGFP cells using 30 mM 2-deoxyglucose/2 μ M rotenone/2 μ g/ml oligomycin for 1 hour at 37 $^{\circ}$ C, as we previously reported (Levine et al., 1993). Studies were performed in NHE3585-EGFP cells because the latrunculin B effect occurred equally on full-length NHE3 and this truncated form, and study of NHE3585 allowed us to focus on the direct cytoskeleton interaction with NHE3, rather than that via NHERF1/NHERF2. The mobility of NHE3 in these cells was significantly decreased by ATP depletion (M_f , 66.4 \pm 2.2%; D_{eff} , (2.5 \pm 0.3) $\times 10^{-10}$ cm²/second in the presence of ATP and M_f^* , 25.1 \pm 3.7%; D_{eff}^* , (9.0 \pm 1.2) $\times 10^{-10}$ cm²/second in ATP depleted cells. * P <0.01, n =6), which is consistent with both myosin motor and actin polymerization involvement in NHE3 mobility.

M_f of YFP-GL-GPI is not dependent on NHERF2 and is not altered by latrunculin B

To determine whether the effects of NHERF2 and actin

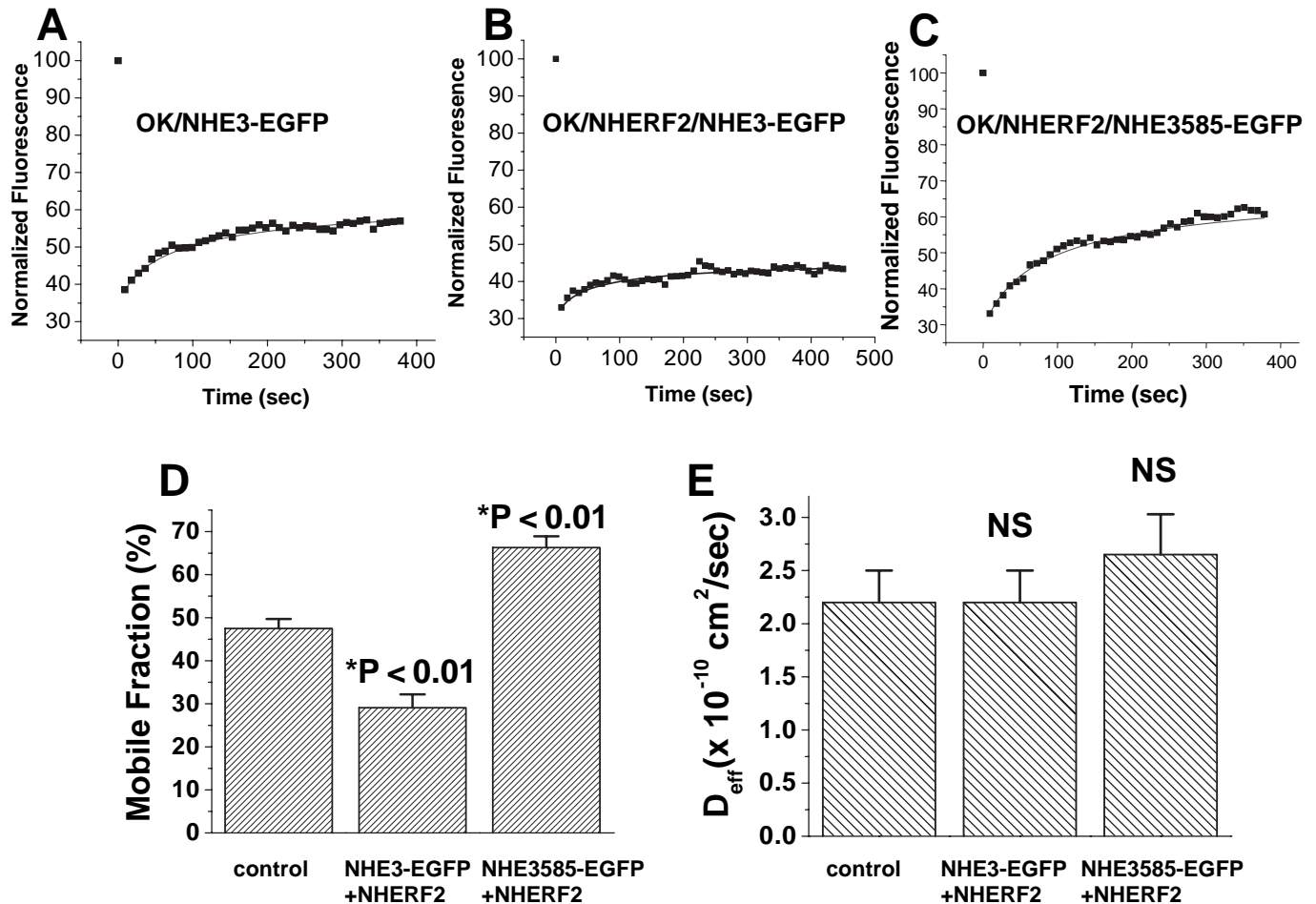


Fig. 8. Effect of PDZ domain proteins NHERF1/2 on the apical surface lateral mobility of non-JN NHE3-EGFP. Photobleaching experiments were done on OK/NHE3-EGFP (A), OK/NHERF2/NHE3-EGFP (B) and OK/NHERF2/NHE3585-EGFP cells (C). Initial fluorescence was set to 100%. The estimated mobile fractions and effective diffusion coefficients are shown in (D) and (E). *P* values are in comparison with OK/NHE3-EGFP (unpaired *t*-test). *n*=21 for OK/NHE3-EGFP, *n*=11 for OK/NHERF2/NHE3-EGFP and *n*=9 for OK/NHERF2/NHE3585-EGFP cells from at least three separate experiments. NS, not significant.

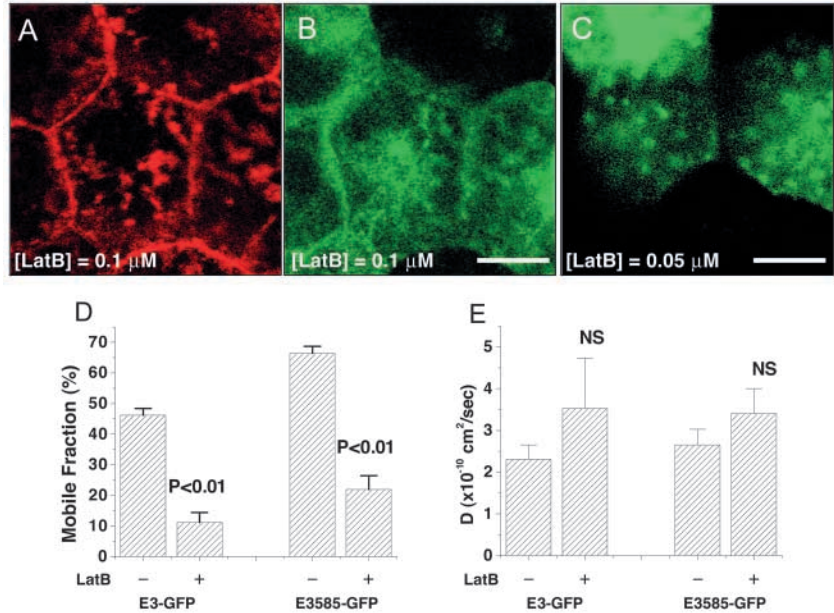
cytoskeleton disruption were specific to NHE3, we examined their effects on the diffusional mobility of an apical plasma membrane protein, YFP-GL-GPI. Because it is GPI-anchored, YFP-GL-GPI should not interact directly with either the actin cytoskeleton or PDZ domain proteins. Further studies were performed with OK and OK/NHERF2 cells, which were transiently transfected with YFP-GL-GPI. These cells showed a microvillus location of the YFP-GL-GPI (Fig. 10A). Fluorescence recovery was determined in these cells under basal conditions and after treatment of OK/NHERF2 cells with latrunculin B (0.05 μ M, 30 minutes), as described above. Compared with NHE3-EGFP, YFP-GL-GPI had a greater M_f which did not significantly differ, on the basis of the presence of NHERF2 (Fig. 10B). Moreover, in spite of latrunculin B-induced cytoskeletal changes, the YFP-GL-GPI M_f was not significantly changed (Fig. 10B). These results show that the NHERF2 and latrunculin B-dependent changes in the NHE3 M_f in OK cells were specific to the extent that they did not occur in a similarly transiently transfected GPI-anchored apical membrane protein in the same cells.

Discussion

BB NHE3 in intestinal and renal proximal tubule cells is acutely regulated as part of digestive and renal physiology and pathophysiology by changes in rates of endocytosis and exocytosis. This regulation involves trafficking under basal conditions as well as increased clathrin-dependent endocytosis to inhibit NHE3 and increased phosphoinositide 3-kinase-dependent exocytosis to stimulate NHE3. In the stimulated endocytosis, NHE3 moves from microvilli to the intervillus clefts where it takes part in clathrin-dependent endocytosis, while opposite movement is involved in stimulated exocytosis. Basal and stimulated exocytosis of NHE3 appears to involve lipid rafts (Li et al., 2001). The current studies used FRAP to assess the lateral mobility of NHE3 in microvilli in the apical membrane of living OK cells to further provide insights in acute NHE3 regulation. NHE3 is bound to NHERF1 and NHERF2 and to the actin cytoskeleton independently of NHERF1/2 (see below), and NHE3 regulation is dependent on an intact cytoskeleton especially via the small GTPase RhoA (Szasz et al., 2000; Szasz et al. 2001). Consequently the effects of NHERF1/NHERF2 and an intact cytoskeleton were

Fig. 9. Effects of latrunculin B on the mobility of NHE3-EGFP and NHE3585-EGFP. OK cells transfected with NHE3-EGFP or OK/NHERF2 cells transfected with NHE3585-EGFP were incubated with latrunculin B (0.1 or 0.05 μM) at 37°C for 30 minutes. Latrunculin B (1 μM , 30 minutes) completely abolished the presence of microvilli (data not shown). F-actin distribution at the apical microvilli was partially disrupted with 0.1 μM latrunculin B for 30 minutes (A). In most of the cells, the apical NHE3-EGFP moved intracellularly as shown in (B). Cell exposed to latrunculin B (0.05 μM for 30 minutes) still had microvilli and NHE3-EGFP remained in the microvilli (C). OK/NHE3-EGFP cells exposed to latrunculin B (0.05 μM) for 30 minutes at 37°C had fluorescence recovery analyzed in the apical plasma membrane non-JN and in the intracellular JN domains. There was no change in M_f or D_{eff} of NHE3-EGFP in the intracellular JN domain ($M_f=37.0\pm 8.3\%$ and $D_{\text{eff}}=(3.9\pm 1.3)\times 10^{-10}$ $\text{cm}^2/\text{second}$; $n=6$). Apical non-JN NHE3-EGFP had decreased M_f compared with cells not treated with latrunculin B (D, left). Similar studies of apical surface non-JN NHE3585-EGFP in

OK/NHERF2/NHE3585-EGFP cells also showed a large decrease in mobile fraction (M_f) caused by this latrunculin B treatment (D, right). The effective diffusion coefficients with and without latrunculin B (0.05 μM) are shown in (E) and did not significantly change with latrunculin B. Data are mean \pm s.e.m. ≥ 7 cells studied for latrunculin B treatment in D and E.



studied on NHE3 lateral mobility. Interaction with NHERF1 and NHERF2 decreased the mobility of NHE3 in an additive manner, with the mobile fraction increasing from ~30% when NHE3 could bind to both NHERF1 and NHERF2, to 47% when only NHERF1 was present to ~65% when a mutant NHE3 (NHE3585) that could bind neither NHERF1 nor 2 was studied. The best explanation for why NHERF1 and NHERF2 have additive effects on NHE3 lateral mobility is that NHE3 exists in large complexes in which NHE3 binds to both NHERF1 and NHERF2 either directly or indirectly. How large are NHE3 complexes? We reported that in the apical membrane of OK cells, NHE3, which as a monomer has a size of ~87 kDa, sediments by gradient centrifugation with size standards of 400-1000 kDa, while in renal proximal tubule Biemesderfer et al., reported that NHE3 is in complexes of ~400 kDa, and in ileal BB NHE3 occurs in complexes of ~500-1000 kDa (X. Li and M.D., unpublished). This makes it likely that in OK apical membranes NHE3 is in complexes of multiple molecules of NHE3, some of which are bound to NHERF1 and others to NHERF2, as well as to other components of the complexes. In OK cells, NHERF1 and NHERF2 are distributed in the apical membrane like they are in Caco-2 cells and ileal BB, and thus the findings presented here are likely to be representative of the situation in intestinal and proximal tubule Na^+ absorptive cells. These studies have been based on effects on NHE3 mobility that occur when NHERF2 is stably expressed in OK cells, which includes an apical membrane pool of NHERF2, and the fact that NHE3 mobility was altered by truncation to aa 585 which is N-terminal of the NHERF1/NHERF2 binding domain, but was not altered by truncation to aa 660 which includes the NHERF1/NHERF2 binding domain (Yun et al., 1997) (B.C. and M.D., unpublished). It is worth noting that since this manuscript was submitted, it has been suggested that the C-

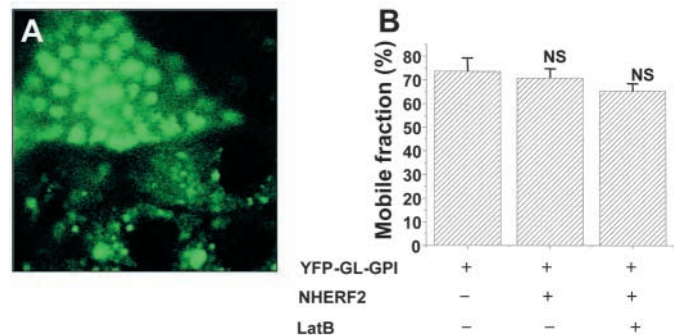


Fig. 10. M_f of OK/YFP-GL-GPI is not dependent on NHERF2 expression and is not altered by latrunculin B. FRAP study was performed with OK and OK/NHERF2 cells transiently transfected with YFP-GL-GPI. (A) The YFP-GL-GPI was present in microvilli in both OK/NHERF2/YFP-GL-GPI and OK/YFP-GL-GPI (not shown) cells. (B) The mobile fractions of microvillus YFP-GL-GPI were estimated in OK/YFP-GL-GPI, OK/NHERF2/YFP-GL-GPI and OK/NHERF2/YFP-GL-GPI + latrunculin B (0.05 μM , 30 minutes) as described above. There was no significant change in M_f of YFP-GL-GPI in OK cells stably expressing NHERF2 and latrunculin B-treated cells. EGFP settings of photobleach, excitation (488 nm) and emission (515 nm) were used for the YFP studies.

terminal 4 amino acids of NHE3 are involved in its binding to NHERF2 in yeast but not in mammalian cells, which would be consistent with our findings (Weinman et al., 2003).

This restriction of lateral mobility of NHE3 with no change in the effective diffusion coefficient associated with NHERF1 and NHERF2 binding is similar to the effects of the PDZ protein PSD-95 on the lateral mobility of the neural channel Kv1.4 expressed in non-polarized HEK293 cells (Burke et al., 1999). Transfection of PSD-95 decreased the mobile fraction

of Kv1.4, while the mobile fraction was increased in Kv1.4 truncated to lack its C-terminal PDZ binding domain. In addition, these manipulations of PSD-95/Kv1.4 interactions led to changes only in the mobile fraction of Kv1.4 and did not alter the diffusion coefficient. Thus it is likely that PDZ domain proteins that bind plasma membrane transport proteins generally provide an anchoring function under basal conditions. Unknown is what other effects on NHE3 handling this anchoring function of PDZ domain containing proteins confers (longer half-life, intracellular localization, changes with signal transduction, etc.).

Somewhat surprisingly, disrupting the actin cytoskeleton using latrunculin B, which prevents actin from polymerizing at its advancing edge, led to a decrease and not an increase in NHE3 lateral mobility. As a negative control, a GPI anchored protein, which is not linked to cytoskeleton or PDZ binding protein demonstrated no effect of cytoskeletal disruption. The cytoskeleton is known to have functions both in fixing proteins in the membrane and in allowing mobility both by anchoring to motor proteins and by filament assembly. The domain of the NHE3 C-terminus involved in this aspect of cytoskeletal function was 5' of the NHERF1/2 binding domain, since latrunculin B caused similar quantitative effects on both wild-type NHE3 and NHE3585 which does not bind NHERF1/NHERF2. Thus there is some cytoskeletal attachment to NHE3 separate from its indirect attachment to cytoskeleton via NHERF1/2. Moreover, this cytoskeletal dependence of the lateral movement of NHE3 in the apical membrane microvilli is consistent with involvement of actin filament assembly rather than simple diffusion, the latter which is unlikely to have been affected by latrunculin B or cytochalasin D.

Actin could be involved in movement of NHE3 via several mechanisms, most prominently via actin assembly vs via attachment to motor proteins such as myosin. There has been a similar debate about the mechanism of involvement of actin in endocytosis. Recently, studies in yeast strongly supported involvement of actin assembly as the mechanism of actin involvement in endocytosis, at least in yeast (Kaksonen et al., 2003). The ATP dependence of NHE3 mobility in OK cell apical membrane, does not allow elimination of either of these mechanisms of cytoskeletal involvement in NHE3 movement.

There are, however, other explanations for the latrunculin B effects on the NHE3 lateral mobility. For instance, it is also possible that actin anchoring may provide connectivity among microvilli, with recovery of NHE3 post-bleaching, which requires NHE3 movement across multiple microvilli, requiring this physical association among microvilli. This dependence of the mobile fraction of apical membrane NHE3 on cytoskeleton is different from what occurs with the intracellular JN NHE3 fluorescence recovery, which was not altered by latrunculin B. This is consistent with the intracellular juxtannuclear pool of NHE3 not appearing to be actin associated based on IF studies (see Fig. 7D3).

Do these biophysical studies have relevance to physiologic regulation of NHE3? In epithelial cells, both stimulation and inhibition of NHE3 are dependent on the binding of NHE3 to NHERF2 and NHERF1 (stimulation by LPA, inhibition by cAMP, cGMP, elevated Ca^{2+}), and at least in fibroblasts, cAMP regulation is dependent on the cytoskeleton (Szasz et al., 2001; Cha et al., 2001; Kim et al., 2002). Moreover, in epithelial cells, most rapid stimulation and inhibition of NHE3 occurs

by changes in trafficking (endo/exocytosis) with varying contributions of independent changes in turnover number. Since changes in endocytosis requires movement of NHE3 from the microvilli to the intervillus clefts and stimulation of exocytosis delivers NHE3 to the intervillus clefts from which it moves to the microvilli, we hypothesize that the demonstrated effects of NHERF1/NHERF2 and the cytoskeleton on lateral mobility of NHE3 in the apical membrane may be relevant to rapid physiologic regulation of NHE3 activity. For instance, stimulated endocytosis may require changing NHE3 lateral mobility by involvement of a myosin motor and may require changes in association with NHERF1/2.

Our initial studies determined whether fluorescence recovery occurred independently of trafficking of NHE3 from intracellular pools. Trafficking from intracellular pools did not appear to significantly contribute to fluorescence recovery for apical membrane NHE3 that was not physically located close to the intracellular juxtannuclear domain, since there was minimal recovery after exposure to the water soluble cross-linking agent BS³. However, apical NHE3 over the juxtannuclear domain did exhibit fluorescence recovery in spite of exposure to BS³, suggesting a contribution of trafficking to recovery of this pool of NHE3. Importantly, the fact that juxtannuclear NHE3 appears to traffic preferentially to the apical NHE3 above the intracellular juxtannuclear domain, demonstrates that trafficking preferentially occurs between the recycling endosomal NHE3 pool and a specific apical membrane domain rather than uniformly to the apical surface. That all NHE3 on the apical surface is not equivalent is a new observation and suggests a 'site' that NHE3 initially arrives at as part of recycling. Previously, Oberleithner et al., showed that in renal epithelial cells, renal Na and K channels had a preferential central apical location (Oberleithner et al., 1992; Schwab et al., 1995). We previously suggested there was a similar preferential central apical location for NHE3 in some OK cells (Akhter et al., 2002). We now suggest that this preferential localization in the apical membrane may be due to preferential targeting from the recycling compartment. Identification of central apical membrane targeting signals to which transporters preferentially traffic would give further insights in normal mechanisms of trafficking. Of note, NHE3 does not have recognized apical membrane targeting signals (it is neither GPI-anchored nor glycosylated), although a portion of NHE3 is present in lipid rafts, and that appears involved in increased trafficking with EGF or clonidine (Li et al., 2001). Thus, we speculate that the NHE3 apical targeting mechanism is lipid raft associated. This conclusion however, must await confirmation in non-overexpressing NHE3 cells, to eliminate that this directed targeting is due to the NHE3 overexpression. Furthermore, blocking PI3-kinase with LY294002 decreased ~50% of apical NHE3 activity and amount without altering the mobile fraction of NHE3 assessed by FRAP in the apical non-juxtannuclear domain. This suggests that total apical membrane NHE3 and that remaining 30 min after inhibiting most trafficking via the exocytic pathway have similar mobile fractions. This is supported by the similar fluorescence recovery demonstrated for the non-JN and above JN apical NHE3 pools, all of which suggest that the preferentially central apical targeted NHE3 has similar lateral mobility to the rest of the apical NHE3.

Could the consequences of NHE3585 expression decreasing NHERF2 location in the apical membrane of OK cells been predicted? Information on mutating NHEs to alter cytoskeletal association come from studies of Denker et al., using NHE1 expressed in PS120 fibroblasts. NHE1 binding to ezrin was necessary for cytoskeletal formation and rearrangement and fibroblast mobility even though NHE1 activity was not necessary for this cytoskeletal organizational function (Denker et al., 2000; Denker and Barber, 2002). Does NHE3 binding to NHERF2 similarly help organize the relevant cytoskeleton, in this case the apical membrane BB? Expressing NHE3 truncated to aa 585, which did not bind NHERF1 or NHERF2, must be analyzed realizing that these cells had a very small amount of endogenous full length OK NHE3, even though we carried out multiple rounds of acid suicide. The acid suicide greatly reduced the rate of endogenous apical Na^+/H^+ exchange (Fig. 1), and only small amounts of opossum NHE3 were detected by immunoblotting (Akhter et al., 2002). In OK/NHE3585 cells, the apical membrane appeared intact, including presence of microvilli with NHE3585 localized to the microvilli and JN regions. In contrast, in OK/ NHE3585 cells, NHERF2 no longer associated with the apical membrane as judged by confocal microscopy (Fig. 7E2). This finding indicates that PDZ proteins can be held in the apical membranes of epithelial cells by their binding partners. Our studies were with OK cells with overexpressed NHERF2 and NHE3. Since NHERF2 binds multiple proteins in the apical membranes of epithelial cells, we predict that it is the total NHERF2 binding to these apical proteins that determines the extent of apical surface NHERF2. This concept probably explains the normal apical location of NHERF1 in OK/NHE3585 cells. NHERF1 binds both NHE3 and endogenous NaPi_2 in OK cells and proximal tubule. In contrast, studies in NHERF1 knock out mice demonstrated that NHERF2 is not able to anchor NaPi_2 to the apical membrane (Wade and Weinman, 2003). We suggest that the apical location of NHERF1 in OK cells expressing NHE3585 is due to the apical presence of additional NHERF1 binding partners that fail to bind NHERF2.

In a study that appeared 'in press' during the second review of this manuscript, CFTR mobility was evaluated in non-polarized MDCK cells using FRAP (Haggie et al., 2004). The results contrast with our findings with NHE3. Unlike NHE3, CFTR had an M_f of at least 90%, indicating that it is not stably anchored to the cytoskeleton. CFTR did, however, interact at least transiently with the cytoskeleton which appears to have a very rapid off rate that allows recovery from the bleach. This occurs via PDZ-domain interactions, as D_{eff} increased when the C-terminal three aa were truncated or mutated so as not to bind PDZ domains. In contrast, NHE3 demonstrated a more restricted M_f when binding domains for NHERF1/NHERF2 are present than in their absence. This is consistent with a tighter, less dynamic interaction of NHE3 with the cytoskeleton than was true for CFTR. Similar to results with NHE3 in latrunculin B treated cells, cytochalasin D treatment decreased CFTR mobility. However, unlike the results with NHE3, the cytochalasin D effect on CFTR was dependent on the presence of the PDZ binding domain of CFTR. These differences may be due to the fact that NHE3 is anchored to the cytoskeleton at domains in addition to those at which the NHERFs bind.

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