Basic Fibroblast Growth Factor Stimulates Surface Expression and Activity of Na\(^{+}/\)H\(^{+}\) Exchanger NHE3 via Mechanism Involving Phosphatidylinositol 3-Kinase*

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Na\(^{+}/\)H\(^{+}\) exchanger NHE3 is a plasma membrane (PM) protein, which contributes to Na\(^{+}\) absorption in the intestine. Growth factors stimulate NHE3 via phosphatidylinositol 3-kinase (PI3-K), but mechanism of this process is not clear. To examine the hypothesis that growth factors stimulate NHE3 by modulating NHE3 recycling, and that PI3-K participates in this mechanism, we used PS120 fibroblasts expressing a fusion protein of NHE3 and green fluorescent protein. At steady state, ~25% of cellular NHE3 content was expressed at PM. Inhibition of PI3-K decreased PM expression of NHE3, which correlated with retention of the exchanger in recycling compartment. In contrast, basic fibroblast growth factor (bFGF) increased PM expression of NHE3, which was associated with a 2-fold increase in rate constant for exit of the exchanger from the recycling compartment. Qualitatively similar effects of bFGF were observed in cells pretreated with PI3-K inhibitors, but their magnitude was only ~50% of that in intact cells. These data suggest that: (i) bFGF stimulates NHE3 by increasing PM expression of the exchanger; (ii) PI3-K mediates PM expression of NHE3 in both basal and bFGF-stimulated conditions, and (iii) not all of the effects of bFGF on NHE3 expression are mediated by PI3-K, suggesting additional regulatory mechanisms.

In the mammalian intestine, sodium and water are reabsorbed by multiple mechanisms which include the activity of Na\(^{+}/\)H\(^{+}\) exchanger NHE3. This transmembrane protein is expressed in the epithelium of renal tubules, intestine, gall bladder, and salivary gland, where it was localized to the apical microvillar domain and, at least in the kidney and in the intestine, to an yet undefined cytoplasmic compartment (1-4). In the small intestine, NHE3 participates in neutral NaCl absorption, and in the increase in Na\(^{+}\) absorption that occurs during neurohormonal stimulation after meals (5). The activity of NHE3 is acutely regulated by multiple mechanisms involving growth factors and protein kinases (6). We and others have shown that stimulation of NHE3 activity by growth factors, okadaic acid, and serum occurs via an increase in the maximal velocity \(V_{\text{max}}\) of the exchange, whereas phorbol ester and carbachol inhibits NHE3 via a decrease in \(V_{\text{max}}\) (6). These effects were observed in non-polarized mesenchymal cells as well as in epithelial cells, and they suggested that at least part of the acute regulation might be accomplished by rapid changes in the number of active exchanger molecules at the plasma membrane.

Over the last few years, a growing body of evidence has indicated that NHE3 might, indeed, be regulated by redistribution of the exchanger molecules between the cytoplasm and the plasma membrane. Thus, recycling of NHE3 has been suggested in kidney epithelial cells based on the results of subcellular fractionation experiments (7, 8), and on the presence of an intracellular compartment accumulating NHE3 (1). Moreover, the protein kinase C-mediated inhibition of endogenous NHE3 in the human colonic adenocarcinoma cell line Caco-2 was reported to involve translocation of the exchanger from brush border into an yet undefined subapical cytoplasmic compartment (9). Recently, D’Souza and colleagues (10) provided the first direct evidence for constitutive recycling of NHE3. These investigators used AP-1 cells expressing rat NHE3 to show that the exchanger molecules recycled between plasma membrane and a juxtanuclear cytoplasmic compartment, and that the latter most probably represented the recycling endosomal compartment. The same laboratory also provided evidence that the constitutive recycling of NHE3 in AP-1 cells was dependent on phosphatidylinositol 3-kinase (PI3-K) activity, and that PI3-K predominantly controlled the exocytic arm of recycling (11). These observations suggested some degree of similarity between NHE3 and the family of other membrane proteins whose constitutive recycling has been shown to be controlled by PI3-K activity. These include the transferrin receptor (12, 13), glucose transporters GLUT4 and GLUT1 (14), and the ATP-dependent canicular transporters in the liver (15). Some evidence suggest that PI3-K activity might be essential not only for control of constitutive activity, but also for mediation of the stimulation of NHE3 by growth factors. Our laboratory reported recently that inhibition of PI3-K with wortmannin eliminated the stimulatory effects of epidermal growth factor (EGF) on the

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‡ The abbreviations used are: NHE3, Na\(^{+}/\)H\(^{+}\) exchanger isoform 3; bFGF, basic fibroblast growth factor; eGFP, red-shifted variant of green fluorescent protein; EGF, epidermal growth factor; GFP, green fluorescent protein; JNC, juntaucluaneous cytoplasmic compartment; PFR, polymerase chain reaction; PM, plasma membrane; PI 3-K, phosphatidylinositol 3-kinase; RBC, recycling endosomal compartment; TF, transferrin; TR, transferrin receptor; TMA, tetramethylammonium; TX-Tf, Texas Red-conjugated human transferrin; VSVG, vesicular stomatitis G protein.

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**MATERIALS AND METHODS**

**Engineering of NHE3-GFP Expression Vector**—To express the NHE3-GFP fusion protein in PS120 cells, we used a cDNA coding for rabbit NHE3-VSVG protein and the red-shifted variant of green fluorescent protein (eGFP) from *Aequorea victoria*. The reason for choosing NHE3-VSVG (NHE3 fused at C terminus with vesicular stomatitis virus [VSVG] epitope tag) was to introduce a spacing sequence between the C terminus of NHE3 and N terminus of GFP. The cellular distribution and activity of the fusion protein closely resembled that of NHE3 lacking the GFP tag, confirming usefulness of this model for the intended studies. Changes in the intracellular steady-state distribution and kinetics of recycling of the fusion protein were examined in living cells using laser confocal microscopy and a novel confocal morphometric analysis.

**Cell Culture and Stable Expression of NHE3-GFP**—Chinese hamster lung fibroblasts (PS120 cells) deficient in endogenous Na⁺/H⁺ exchangers (gift from Dr. J. Pouyssegur) were cultured in DMEM supplemented with 0.1 mM nonessential amino acids, 1 mM pyruvate, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum, in a 10% CO₂ humidified incubator at 37 °C. Cells were transfected with NHE3-GFP cDNA (or with pEGFP-N3 vector) using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. The cell line expressing NHE3-GFP was subsequently isolated using dilutional cloning, propagated in a large scale culture, and frozen in liquid nitrogen. In all experiments, transfected cells were used during the initial three passages after being thawed from the frozen stock.

**FIG. 1.** Linear representation of the NHE3-VSVG construct inserted into the multiple cloning site (M) of pEGFP-N3 vector in-frame with 5’ end of eGFP sequence. The inserted cDNA molecule contained the full sequence coding for 832 amino acids (aa) of rabbit NHE3, followed by a 8-aa-amino acid spacer sequence (SP) and a 11-amino acid vesicular stomatitis virus G protein epitope sequence (VSVG). The SaI restriction site replaced the stop codon originally present at the 3’ end of the NHE3-VSVG sequence.

**Na⁺ reabsorption in the rabbit ileum, and on Na⁺/H⁺ exchange in Caco-2 cells (16). Based on these results, it was tempting to speculate that the PI 3-K-mediated mechanism by which growth factors stimulate NHE3 activity involves changes in the dynamics of recycling of NHE3, which, in turn, results in an increased expression of the exchanger at the PM. Such mechanism could, theoretically, be based on the same signal transduction pathway(s) as the constitutive regulation of the exchanger. In such situation, regulatory stimulus might simply amplify the existing mechanisms (e.g. by stimulating the activity and/or intracellular redistribution of PI 3-kinase). Alternatively, the growth factor-regulated pathway might also involve specific mechanisms different from those utilized by the constitutive pathway.

In this report we present data suggesting that bFGF stimulates NHE3 activity by increasing expression of the exchanger at the PM. We also provide evidence suggesting that the increased expression results from increased rate of exit of the exchanger molecules from the recycling endosomal compartment and that this mechanism is partially dependent on PI 3-K activity. In order to correlate changes in cellular distribution of NHE3 with Na⁺/H⁺ exchange rate, we engineered and stably expressed in PS120 fibroblasts a fusion protein of rabbit NHE3 and green fluorescent protein (GFP). The cellular distribution and activity of the fusion protein closely resembled that of NHE3 lacking the GFP tag, confirming usefulness of this model for the intended studies. Changes in the intracellular steady-state distribution and kinetics of recycling of the fusion protein were examined in living cells using laser confocal microscopy and a novel confocal morphometric analysis.

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**Measurement of Na⁺/K Activity—**The rate of Na⁺/K exchange in the transfected cells was examined using a modification of the previously described method based on confocal microscopy and SNARF-1 as a pH-sensitive fluorescent indicator (19). PS120-E3G cells cultured on glass coverslips (~75% confluence) were serum-starved for 3–4 h, and incubated for 30 min at room temperature in Na⁺ medium (90 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 0.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, 25 mM glucose, 20 mM HEPES, pH 7.4) containing 15 μM SNARF-1 (5-(and-6)-carboxy SNARF-1, acetoxymethyl ester, acetyl; Molecular Probes, Eugene, OR) and 40 mM NH₄Cl (to promote subsequent intracellular acidification). Cells were initially perfused with tetramethylammonium (TMA) medium (similar to Na⁺ medium, except that Na⁺ salts were replaced by TMA salts), which was then switched to Na⁺ medium containing 90 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 0.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, 25 mM glucose, 20 mM HEPES, pH 7.4.

**Quantitation of Cellular Distribution of NHE3-GFP**—To examine the distribution of NHE3-GFP in PS120-E3G cells we developed a morphometric method based on confocal microscopy and digital image processing, which we describe in detail elsewhere (2). The principle of this method was to label the plasma membrane (PM) of the examined living cells with a vital fluorescent dye, and then to digitally subtract the areas corresponding to the membrane from the serial confocal images of the GFP fluorescence in the examined cells. To label the PM for studies of cellular distribution of NHE3, and also for examination of endo- and exocytic events of NHE3-GFP in living cells, we used a fluorescent dye FM 4–64 (Molecular Probes, Eugene, OR). PS120-E3G cells cultured on glass coverslips were perfused on the microscope stage at 4 °C with Na⁺ medium containing FM 4–64 (20 μM) for 1 min, and serial optical sections in the xy plane were collected in steps of 0.4 μm in the z axis across the entire cell. The excitation wavelength was set at 488 nm, and fluorescent images were collected simultaneously at 510 nm (eGFP) and 640 nm (FM 4–64). The stored images were subsequently analyzed using MetaMorph software (Universal Imaging Corp.) as follows. First, the images of FM 4–64 fluorescence were binarized and reversed. Next, a new series of images was generated by performing a Boolean operation "AND" in the pairs of images representing FM 4–64 and eGFP signals within the same optical section. The result of this operation was a series of images in which the eGFP signal overlapping with the FM 4–64 signal was digitally subtracted. Final quantification of the PM content of NHE3-GFP (expressed as percent of total cellular content) was performed using formula: 100 * (IFIT₁ - IFIT₂) / IFIT₁ × 100, where IFIT₁ and IFIT₂ stand for total cellular and cytoplasmic eGFP fluorescence intensity, respectively, and n stands for the number of optical sections required to scan the entire cell.

To complement data obtained by the morphometric analysis, we quantitated the cellular distribution of NHE3-GFP using cell surface biotinylation. PS120-E3G cells grown to ~75% confluence in 10-cm Petri dishes were rinsed several times with phosphate-buffered saline followed by borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl₂, 10 mM H₂BO₃, pH 9.0). The entire procedure was performed at 4 °C unless otherwise indicated. Cells were then incubated twice for 20 min at 4 °C with 3 μl of borate buffer containing 1.5 mg of NHS-SS-biotin (Pierce; biotinylation solution), followed by incubation with quenching buffer (120 mM NaCl and 20 mM Tris, pH 7.4). Cells were then scraped, solubilized by guest on July 26, 2018http://www.jbc.org/Downloaded from
in 1 ml of lysis buffer (150 mM NaCl, 3 mM KCl, 5 mM EDTA trisodium, 3 mM EGTA, 1% Triton X-100, 60 mM HEPES, pH 7.4), and sonicated for 20 s. The lysates were agitated for 30 min and spun at 12,000 × g to remove insoluble debris (total fraction). The supernatant was incubated with avidin-agarose, spun, and the remaining supernatant was retained as the intracellular fraction. The avidin-agarose beads were boiled in Laemmli sample buffer yielding the surface fraction. Western analysis was performed on dilutions of all three fractions run on the same gel. Separated proteins were transferred to nitrocellulose, and probed with monoclonal anti-VSVG antibody F5D4 (hybridoma culture medium at 1:5 dilution; kindly provided by Dr. D. Louvard, Curie Institute, Paris, France), or polyclonal anti-GFP antibody (1:1,000; CLONTECH, Palo Alto, CA). Bands were visualized using enhanced chemiluminescence and quantified using a densitometer and Image-quant software.

**Examination of Kinetics of NHE3-GFP Recycling**—To examine the dynamics of NHE3-GFP entry into juxtanuclear cytoplasmic compartment (JNC), the PS120-E3G cells on glass coverslips were perfused in Na+ medium containing FM 4–64 (20 μM). Cells were scanned (serial 0.4-μm optical sections in xy axis) every 2–3 min, with signals from eGFP (510 nm) and FM 4–64 (640 nm) collected by separate photomultipliers. Analysis of yellow-orange particles colocalizing both fluorophores (vesicles derived from the PM containing FM 4–64 and NHE3-GFP) in the cytoplasm and in JNC was performed in the stored images using MetaMorph software. First, the JNC area was defined by selecting contiguous 6 × 6 pixel areas containing >50% of pixels with intensity of GFP signal higher then the set threshold. This approach let us detect the border of JNC within a zone of 0.3 μm (~3 pixels). In the JNC defined this way, more than 90% of all pixels colocalized with internalized Texas Red-conjugated transferrin (TX-Tf), a relatively specific marker of recycling endosomal compartment. Next, the particles outside JNC were counted in three categories: (i) containing only eGFP; (ii) containing only FM 4–64, and; (iii) containing both eGFP and FM 4–64. Within JNC area, the intensity of FM 4–64 signal was quantitated on a pixel to pixel basis, separately for pixels colocalizing and not colocalizing with eGFP. The values obtained for each optical section were then summed, yielding the integrated fluorescent intensity of eGFP in the PM-derived vesicles accumulated in peripheral cytoplasm and in the JNC.

To evaluate the dynamics of exit of NHE3-GFP from JNC, the PS120-E3G cells were first perfused in the presence of FM 4–64 (20 μM) for 30 min at 33 °C to saturate JNC with PM-derived, NHE3-GFP-containing vesicles. Next, the cells were perfused with FM 4–64-free medium, which resulted in a dissociation of FM 4–64 from the FM within ~90 s. Following perfusion for 3 min (to chase any remaining endosomal vesicles to the JNC), serial images were collected as described above. The fading fluorescence of FM 4–64 within the area of JNC was quantitated in the stored images as described above, separately for pixels colocalizing and not colocalizing with eGFP.

**Colocalization of eGFP with Intracellular Transferrin Receptor**—To visualize the recycling endosomal compartment in PS120-E3G cells, the cells on glass coverslips were incubated for 30 min at 33 °C with 25 μg/ml TX-Tf (Molecular Probes, Eugene, OR) in the presence or absence of an excess of uncojugated transferrin (1 mg/ml; Sigma). To promote up-regulation of expression of transferrin receptor (TR) in PS120-E3G cells, the cells were cultured for 24 h in the presence of 5% fetal bovine serum and 4 μg/ml deferoxamine (Sigma). Cells were then fixed with 2% paraformaldehyde at 4 °C for 10 min, washed with phosphate-buffered saline, and examined using confocal fluorescent microscopy. The excitation/emission wavelengths were set at 488/510 nm. For colocalization with transferrin receptors, PS120-E3G cells were incubated for 30 min in the absence (A and B) or presence (C and D) of wortmannin, which was followed by an additional 30 min of incubation with TX-Tf. Cells were then lightly fixed with paraformaldehyde and examined under confocal microscopy. The high degree of colocalization of juxtanuclear accumulation of NHE3-GFP with the steady-state accumulation of internalized TX-Tf in control cells as well as in cells pretreated with wortmannin. Arrow in panel B points at a rare cell which internalized fluorescent transferrin but did not express NHE3-GFP. This is shown to document lack of a crossover of the fluorescent signal from the TX into the GFP channel. Images represent 0.4-μm optical sections in the xy plane. Bars, 10 μm.

**Statistical Analysis**—Numerical data are expressed as means ± S.E., and the significance of difference between experimental groups was analyzed by using the two-tailed Student’s t test.

**RESULTS**

**NHE3-GFP Is Stably Expressed at the Plasma Membrane** and in the Recycling Endosomal Compartment—The pattern of cellular distribution of NHE3-GFP in stably transfected PS120 fibroblasts was examined in three-dimensional reconstructions of images of both living and fixed cells obtained by confocal microscopy. In control conditions, the bulk of NHE3-GFP was divided among three compartments: (i) the PM, (ii) the juxtanuclear accumulation, and (iii) the population of small particles (0.1–0.4 μm) distributed throughout the peripheral cytoplasm (Fig. 2, top). In both living and fixed cells, NHE3-GFP within the juxtanuclear compartment colocalized to high de-
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FIG. 3. Quantitation of cellular distribution of NHE3-GFP by confocal morphometric analysis (top), and by cell surface biotinylation (bottom). Panel A shows a 0.4-μm optical section of a living PS120-E3G cell demonstrating the characteristic distribution of NHE3-GFP between the plasma membrane and the juxtanuclear cytoplasmic compartment. Bar, 20 μm. Panel B shows the binary mask (prior to inversion), which was obtained after labeling of the plasma membrane with FM 4–64. Panel C shows the result of Boolean operation “AND” performed on images in A and B. Note the disappearance of the eGFP fluorescence from the area corresponding to the plasma membrane. For surface biotinylation, PS120-E3G cells were incubated in control medium in the absence (CTR) or in the presence of wortmannin (WT). The cell surface proteins were biotinylated with NHS-SS-biotin and the biotinylated material was separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-VSVG monoclonal antibody. The fluorogram shown was obtained from one representative experiment. Total cell lysates were run in lanes T. Material extracted from avidinagarose beads (surface NHE3-GFP) was run in lanes S, whereas the supernatant from the avidin precipitations (the intracellular fraction) was run on lanes I. Material separated in each lane represents the same number of cells.

NHE3-GFP Is a Functional Na+/H+ Exchanger—The main reason for using SNARF-1 (instead of BCECF, which we have used in the past) as a pH indicator was an overlap of emission maxima for eGFP (510 nm) and BCECF (530 nm). The SNARF-1 method could reliably detect a difference in pH < 0.02 units within the pH range of 6.30–7.60. Moreover, the calibration curve obtained for SNARF-1 was almost identical to the one obtained for PS120-E3G cells using SNARF-1 as pH indicator. Panel B shows dynamics of pH recovery in 5 randomly chosen PS120-E3G cells. Cells were loaded with SNARF-1 and acidified in the absence of Na+, and the Na+-dependent pH recovery was examined using confocal microscopy. Arrow indicates the onset of addition of Na+ (131 mM) into the perfusion buffer.

FIG. 4. Intracellular pH (pH_i) calibration curve for SNARF-1 and rates of Na+-dependent pH_i recovery obtained from individual NHE3-E3G cells. Panel A shows pH_i calibration curves generated in PS120-E3G (solid line) and PS120-E3V (dotted line) cells using SNARF-1 as pH indicator. Panel B shows dynamics of pH_i recovery in 5 randomly chosen PS120-E3G cells. Cells were loaded with SNARF-1 and acidified in the absence of Na+, and the Na+-dependent pH_i recovery was examined using confocal microscopy. Arrow indicates the onset of addition of Na+ (131 mM) into the perfusion buffer.

VSVG molecules were expressed in fusion with eGFP, and that no detectable amounts of NHE3-VSVG free of GFP moiety (M_r ~ 85 kDa) was expressed in the transfected cells (Fig. 3, bottom). Similar results were obtained when the blots were probed with the anti-GFP antibody (data not shown).
Constitutive recycling of NHE3-GFP in living PS120-E3G cells. Cells on glass coverslips were perfused on the confocal microscope stage at 33 °C, and serial images were collected separately for NHE3-GFP (left image in each panel) and for FM 4–64 (right image in each panel). Schematic diagram of the experimental design is shown in the right lower corner (Na, Na+ medium; Na+ + FM, Na+ medium with 20 μM FM 4–64). Panel A shows two cells shortly prior to exposure to FM 4–64 (FM). Note a characteristic distribution of NHE3-GFP among the plasma membrane (PM), the recycling endosomal compartment (REC), and the cytoplasmic particles (arrowheads). Also, note a lack of crossover of GFP signal into FM channel. Three minutes after exposure to FM (panel B), labeling of the PM with the styryl dye is well visible. Note the presence of particles colocalizing eGFP and FM (arrowheads) in the cytoplasm. After 18 min of continuous exposure to FM (panel C), the internalized membrane vesicles containing both fluorophores are saturating the REC area. Note the continuous presence of peripheral particles colocalizing eGFP and FM (arrowheads). Nine minutes after discontinuation of exposure to FM (panel D), the number of particles colocalizing FM and eGFP within REC is visibly declining, which is associated with gradual reappearance in the cytoplasm of multiple particles carrying both fluorophores (arrowheads). Twenty-eight minutes after discontinuation of exposure to FM (panel E), only residual fluorescent signal from FM is visible within the REC area, and no colocalizing particles are seen in the peripheral cytoplasm. All images represent 0.4-μm optical sections from the same two cells. Images were collected with low averaging rate and with attenuated laser power to minimize photodamage. Bar, 20 μm.

Analysis of the kinetics of recycling of FM 4–64/NHE3-GFP vesicles in PS120-E3G cells revealed that both accumulation in, and exit from REC of NHE3-GFP were governed by a single exponential function, thus suggesting first order kinetics. In order to calculate the apparent rate constants for both processes, the kinetic data were normalized for the maximal intensity of the FM 4–64 fluorescence within the REC, and the logarithms of the obtained values were plotted against time (Fig. 6). Slopes of the decay curves yielded the apparent rate constants for accumulation within (k_{in}) and for exit from (k_{ex}) REC (22). In 36 cells analyzed, the average calculated k_{in} was 0.116 ± 0.015, and the average k_{ex} was 0.036 ± 0.004 (means ± S.E.; Table I). Assuming first order kinetics, recycling of NHE3-GFP was governed by equation dC/dt = k_{in}P − k_{ex}C, where P and C stand for concentrations of NHE3-GFP at the PM and in the REC, respectively, and dC/dt represents accumulation of NHE3-GFP in REC (23). Since, at steady state, dC/dt = 0, k_{in}/k_{ex} = C/P. Thus, the ratio of k_{in}/k_{ex}, calculated using the
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**Fig. 6. Kinetics of constitutive recycling of NHE3-GFP in PS120-E3G cells.** To examine the steady-state dynamics of entry of PM-derived NHE3 into REC, the cells were perfused on the confocal microscope stage at 33 °C in the continuous presence of FM 4–64, and images were collected every 2–3 min. The integrated intensity of the FM 4–64 fluorescence colocalizing with eGFP was plotted as log(\(F_t - F_0\)/\(F_t\) × 100) versus time, where \(F_0\) and \(F_t\) represent FM 4–64 fluorescence intensity at saturation (an end point) and at a given time point, respectively (●). For examination of the dynamics of exit of NHE3 from REC, PS120-E3G cells were perfused in presence of FM 4–64 for 30 min, after which time the fluorophore was removed from perfusate, and images were collected every 2–3 min. The integrated intensity of FM 4–64 fluorescence colocalizing with eGFP was plotted as log(\(F_s\)/\(F_0\) × 100) versus time, where \(F_s\) and \(F_0\) represent fluorescence intensity at a given time point and at the time 0 (initial maximal saturation of REC), respectively (▲). Both processes could be best fitted with a single straight line, the slope of which represented the rate constant for internalization (in the example shown \(k_i = 0.117\)) and for exit from REC (in example shown \(k_{ex} = 0.036\), respectively (22). Data for each plot were obtained from a single cell in a representative experiment.

**Fig. 7. Effects of exposure of PS120-E3G cells to bFGF, wortmannin, or wortmannin followed by bFGF.** Cells were incubated at 33 °C in either control medium (CTR), or in medium containing bFGF (bFGF), wortmannin (WT), or wortmannin followed by bFGF (WT+bFGF). For examination of kinetics of accumulation of NHE3-GFP in the recycling endosomal compartment (REC), cells were perfused with FM 4–64 (20 μM) and images were collected every 2 min for up to 30 min. For studies on exit of NHE3-GFP from REC, cells were preincubated with FM 4–64 for 30 min, after which time FM 4–64 was washed out from the plasma membrane, and serial images were collected during subsequent 30 min of perfusion with FM–free medium. The integrated fluorescence intensities of NHE3-GFP colocalizing with FM 4–64 within the area of REC were calculated as described under “Materials and Methods.” Data are means ± S.E. from 56 cells in three separate experiments.

**Table I**

**Kinetic parameters of NHE3-GFP recycling in PS120-E3G cells exposed to bFGF, wortmannin, or wortmannin followed by bFGF.**

| Treatment group | Rate constant of accumulation in REC (min⁻¹) | Rate constant of exit from REC (min⁻¹) |
|-----------------|---------------------------------------------|---------------------------------------|
| CTR             | 0.116 ± 0.015                      0.036 ± 0.004                             |
| bFGF            | 0.115 ± 0.019                      0.072 ± 0.009**                             |
| WT              | 0.160 ± 0.014*                     0.010 ± 0.002**                             |
| WT+bFGF         | 0.163 ± 0.019*                     0.021 ± 0.003***                             |

* significantly different (\(p < 0.05\)) from respective CTR value; **, significantly different (\(p < 0.01\)) from respective CTR value; ***, significantly different (\(p < 0.001\)) from respective WT value.

values of C and P obtained from confocal morphometric analysis equals 3.0. This value is very similar to the actual ratio (3.22) obtained from measured kinetics of NHE3-GFP recycling, supporting the assumption that accumulation of NHE3 in, and exit from REC are governed by first order kinetics.

**PI 3-Kinase Is Involved in Regulation of Basal NHE3 Activity and PM Expression—**To evaluate the role of PI 3-K in main-
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**Comparison of the effects of bFGF on the activity and plasma membrane expression of NHE3-GFP in control PS120-E3G cells and in cells pretreated with PI 3-K inhibitors wortmannin or LY294002**

PS120-E3G cells were preincubated for 30 min at 33°C in plain Na+ medium (CTR+bFGF) or in Na+ medium containing wortmannin (WT+bFGF) or LY294002 (LY+bFGF), after which time bFGF was added to the incubation media for the next 20 min in half of the experimental groups. Measurements of Na+/H+ exchange rate were performed using SNARF-1 and confocal microscopy, and quantitation of the expression of NHE3-GFP at the plasma membrane was performed using confocal morphometric analysis. Results are means ± S.E. from 48 cells in two separate experiments, and represent the absolute increase in the measured parameter observed after addition of bFGF in the respective experimental group. * significantly (p < 0.01) different from respective CTR+bFGF value.

**Table II**

Comparison of the effects of bFGF on the activity and plasma membrane expression of NHE3-GFP in control PS120-E3G cells and in cells pretreated with PI 3-K inhibitors wortmannin or LY294002.

| Experimental group | Increase in Na+/H+ exchange rate | Increase in NHE3 surface expression |
|--------------------|----------------------------------|-----------------------------------|
| CTR+bFGF           | 0.031 ± 0.003                    | 13.0 ± 1.5                        |
| WT+bFGF            | 0.016 ± 0.004*                   | 6.4 ± 0.4*                        |
| LY+bFGF            | 0.018 ± 0.003*                   | 7.3 ± 0.7*                        |

**Fig. 8. Effects of exposure of PS120-E3G cells to bFGF, wortmannin, or wortmannin followed by bFGF on the patterns of cellular distribution (top) and relative surface expression (bottom) of NHE3-GFP.** Prior to imaging cells were incubated in control medium (CTR), or in the presence of bFGF (FGF), wortmannin (WT), or wortmannin followed by bFGF (WT+bFGF). Images of eGFP fluorescence in living cells were obtained by confocal microscopy. Note the apparent increase in the plasma membrane expression of NHE3-GFP in the bFGF-treated cells when compared with controls. Additionally, note the practical disappearance of the fluorescent signal at the plasma membrane, and tubulation and compaction of the recycling endosomal compartment in cells pretreated with wortmannin. Exposure of wortmannin-treated cells to bFGF resulted in a weak but visible increase in the membrane expression of NHE3-GFP, and in a less compacted recycling compartment. Images represent 0.4-μm optical sections obtained in the xy plane. Bars, 20 μm. *, significantly different (p < 0.01) from control group. **, significantly different (p < 0.01) from wortmannin group.

IC_{50} of 25 nM (Fig. 7B). Exposure of PS120-E3G cells to LY294002 resulted in an inhibition of the basal Na+/H+ exchange rate by 65%, a value similar to that observed for wortmannin (Fig. 7C). Quantitatively similar effects of PI 3-K inhibition on the NHE3 activity were observed in PS120-E3V cells (expressing NHE3-VSVG protein lacking the GFP tag), which indicated that the GFP tag did not alter the responsiveness of NHE3 to the regulatory mechanisms involving PI 3-K (Fig. 7C).

The inhibitory effects of PI 3-K inhibitors on NHE3-GFP activity were accompanied by profound changes in the cellular distribution of the fusion protein. We observed a significant decrease of intensity of NHE3-GFP fluorescence at the PM, which was associated with tubulation, compaction, and an overall increase in the fluorescence intensity of the REC (Fig. 8, top). Confocal morphometric analysis revealed that exposure to wortmannin resulted in a significant decrease of the PM expression of NHE3-GFP, from 25 ± 3% of the total cell content in control cells to 7 ± 1% in wortmannin treated cells (Fig. 8, bottom). Similar results were obtained by cell surface biotinylation, where treatment with wortmannin resulted in a decrease of biotinylated (PM) fraction of NHE3-GFP, from ~22% in control cells to ~5% in wortmannin-treated cells (Fig. 3, bottom). This decrease in PM expression of NHE3 was most likely due to an over 3-fold decrease in the k_{ex} and also due to a 1.4-fold increase in k_{in} (Table I).

bFGF Stimulates NHE3 Activity by Increasing PM Expression of the Exchanger—Effects of bFGF on NHE3 activity in PS120-E3G cells are shown in Fig. 7 and Table II. Exposure of PS120-E3G cells to bFGF for 20 min resulted in a significant increase in the rate of Na+ driven pH_{i} recovery. The average increase (change in ΔpH/s) was 0.031 ± 0.003 (60% over the control rate; Table II). Higher concentrations of bFGF (up to 200 ng/ml) did not result in further augmentation of the stimulatory effect of the growth factor (data not shown). This stimulatory effect of bFGF on NHE3-GFP activity was associated with a visible increase in expression of the exchanger at the PM (Fig. 8, top). By confocal morphometric analysis, NHE3-GFP expression at the PM increased from 25 ± 3% of the total cellular content in control cells to 38 ± 2% in bFGF-treated cells (Fig. 8, bottom). Thus, ~13% of the total cellular content of NHE3-GFP was translocated from the cytoplasm into the PM in response to bFGF stimulation. In a separate series of experiments, the entire process of bFGF-induced translocation of NHE3 was evaluated in the same living cells. In these experiments, the bFGF-stimulated increase in PM expression of the fusion protein was accompanied by a quantitatively similar decrease in the amounts of NHE3-GFP accumulated within the REC, despite the observed differences in the initial amounts of NHE3-GFP (data not shown). This stimulatory effect of bFGF on NHE3-GFP activity was associated with a visible increase in expression of the exchanger at the PM (Fig. 8, top). By confocal morphometric analysis, NHE3-GFP expression at the PM increased from 25 ± 3% of the total cellular content in control cells to 38 ± 2% in bFGF-treated cells (Fig. 8, bottom). Thus, ~13% of the total cellular content of NHE3-GFP was translocated from the cytoplasm into the PM in response to bFGF stimulation. In a separate series of experiments, the entire process of bFGF-induced translocation of NHE3 was evaluated in the same living cells. In these experiments, the bFGF-stimulated increase in PM expression of the fusion protein was accompanied by a quantitatively similar decrease in the amounts of NHE3-GFP accumulated within the REC, despite the observed differences in the initial amounts of NHE3-GFP (data not shown). This stimulatory effect of bFGF on NHE3-GFP activity was associated with a visible increase in expression of the exchanger at the PM (Fig. 8, top). By confocal morphometric analysis, NHE3-GFP expression at the PM increased from 25 ± 3% of the total cellular content in control cells to 38 ± 2% in bFGF-treated cells (Fig. 8, bottom). Thus, ~13% of the total cellular content of NHE3-GFP was translocated from the cytoplasm into the PM in response to bFGF stimulation. In a separate series of experiments, the entire process of bFGF-induced translocation of NHE3 was evaluated in the same living cells. In these experiments, the bFGF-stimulated increase in PM expression of the fusion protein was accompanied by a quantitatively similar decrease in the amounts of NHE3-GFP accumulated within the REC, despite the observed differences in the initial amounts of NHE3-GFP (data not shown). This stimulatory effect of bFGF on NHE3-GFP activity was associated with a visible increase in expression of the exchanger at the PM (Fig. 8, top). By confocal morphometric analysis, NHE3-GFP expression at the PM increased from 25 ± 3% of the total cellular content in control cells to 38 ± 2% in bFGF-treated cells (Fig. 8, bottom). Thus, ~13% of the total cellular content of NHE3-GFP was translocated from the cytoplasm into the PM in response to bFGF stimulation.
LY294002. Exposure of wortmannin-pretreated PS120-E3G cells to bFGF resulted in an increase in NHE3-GFP activity when compared with wortmannin-alone group, from 0.020 ± 0.004 to 0.036 ± 0.004 (ΔpH/s; Fig. 7). Similar effect was observed in cells pretreated with LY294002 (Table II). However, the magnitude of the stimulatory effects of bFGF (0.016 [ΔpH/s]) in presence of the PI 3-K inhibitors was only approximately 50% of that observed in cells with intact PI 3-K activity (Table II).

Exposure of wortmannin-pretreated cells to bFGF resulted in a small but visible increase in the PM expression of the fusion protein when compared with wortmannin alone group (Fig. 8, top). By confocal analysis, the steady-state fraction of fusion protein associated with the PM increased from 7 ± 1% (wortmannin alone) to 13 ± 2% (wortmannin + bFGF) of the total cellular NHE3-GFP content. In absolute terms, this increase constituted approximately 50% of that observed in cells with intact PI 3-K activity (Fig. 8 and Table II). Similar effects of bFGF were observed in cells pretreated with LY294005 (Table II). Comparison of the kinetics of NHE3 recycling in PS120-E3G cells exposed to wortmannin followed by bFGF with those obtained for cells exposed to wortmannin alone revealed that the observed effect of bFGF on NHE3-GFP expression at the PM was predominantly due to a 2-fold increase in k_{ex} (Table I).

**DISCUSSION**

Until recently, studies on cellular distribution and recycling of membrane proteins utilized indirect methods including subcellular fractionation, immunoelectron microscopy, or immunolocalization of epitope-tagged molecules of interest. Although these methods have been often applied in an elegant and well controlled fashion, they have several limitations. These include the inability to monitor the real-time trafficking of membrane proteins in living cells, the non-linear stoichiometry of antigen-antibody binding, the difficulty in preventing dissociation of labeled antibodies from antigens in the living cells, and the necessity for cell permeabilization to label the intracellular structures. These limitations have been circumvented, to various degrees, by recent introduction of GFP as an *in vivo* reporter tag. GFP, and especially its recently engineered mutated variants, is brightly fluorescent, relatively resistant to photobleaching, and does not require exogenous cofactors or substrates (24, 25). Importantly, in many cases the GFP tag does not significantly affect biological activity, regulation, or intracellular trafficking of the protein of interest (26). Results presented in this report indicate that C-terminal fusion of NHE3 with the red-shifted variant of GFP (eGFP) did not significantly alter the investigated properties of the exchanger. As shown in Fig. 2, cellular distribution of NHE3-GFP in stably transfected PS120 cells resembled that of NHE3-VSVG protein lacking the GFP tag. Importantly, the magnitude of response to bFGF and PI 3-K inhibitors was very similar in both cell lines (Fig. 7), indicating that fusion with eGFP did not significantly affect the responsiveness of the NHE3 moiety to these regulatory factors. It remains to be determined whether other properties of NHE3, which have not been investigated in our studies, remained intact following fusion with GFP.

Growth factors have been previously shown to stimulate NHE3 in rabbit ileum and in non-epithelial as well as epithelial cell lines (16, 27–29). Since stimulation of NHE3 activity occurred via an increase in maximum velocity (V_{max}) of the exchange, it suggested either a rapid increase in the number of NHE3 molecules at the PM or an increase in the turnover number as a putative underlying mechanisms. In this report, we confirmed the redistribution hypothesis by showing that bFGF stimulated NHE3 activity in PS120 cells by increasing expression of the exchanger at the PM. Moreover, practically all of the bFGF-dependent stimulation of Na^+ /H^+ exchange could be accounted for this increase in surface expression of NHE3. Thus, bFGF stimulated NHE3 activity by ~60%, and the exchanger’s PM expression by ~50% over control values, respectively. These results effectively ruled out a significant change in the turnover number of the individual NHE3 molecules as the underlying mechanism of stimulation. At this point, three major questions concerning the mechanism of such a rapid increase in the PM expression of the exchanger should be addressed: (i) did the observed effect of bFGF result from altered kinetics of NHE3 recycling and, if so, did it result from an increased rate of insertion of NHE3 molecules into PM, a decreased rate of removal of the exchanger molecules from PM, or both; (ii) what was the intracellular source of molecules being inserted into PM, and; (iii) since PI 3-K has been implicated in mediation of the effects of growth factors on NHE3 activity, what was the role (if any) of this kinase in the effects exerted by bFGF on the recycling of NHE3. In regard to the first question, results of studies presented in this report strongly suggest that bFGF increased the PM expression of NHE3 by a selective stimulation of the rate of exit of the exchanger from REC (Table I). Stimulation of exocytosis by growth factors has previously been described for seemingly heterogeneous group of processes like recycling of TIR in human and mouse fibroblasts (30, 31), acrosomal exocytosis in bull spermatozoa (32), movement of exocytic vesicles during formation of membrane ruffles in fibroblasts (33), or an EGF-induced acute increase in brush border surface area in rabbit jejunal epithelium (34). It is not clear at this moment whether all of the above processes, including bFGF-stimulated exocytosis of NHE3, share a common regulatory pathway or whether the regulation is protein- or process-specific. One mechanism leading to higher specificity of regulation might be directing the traffic of regulated pool of the protein away from the constitutive bulk membrane flow. In respect to GLUT4, it has been
suggested that only ~40% of the intracellular GLUT4 molecules shares the same vesicle pool with TIR, whereas the remainder of the transporter molecules is trafficking in a separately regulated vesicle pool (35, 36). It has yet to be determined whether similar phenomenon exists in respect to the growth factor-regulated recycling of NHE3.

In regard to the second question, our data indicated that most, if not all, of the NHE3 molecules arriving at the PM as a result of exposure of PS120-E3G cells to bFGF originated in the JNC (Figs. 5 and 9). In this study, we did not attempt to precisely define the nature of JNC in PS120-E3G cells. However, we did find a high degree of colocalization of NHE3-GFP with the steady-state intracellular accumulation of TX-TTTR complexes. Since TIR is known to accumulate in the recycling endosomal compartment and, therefore, to be a relatively specific marker for this compartment (37), our findings suggested that JNC corresponded to the recycling endosomal compartment. This is in agreement with conclusions drawn by D’Souza and colleagues (10) in regard to a similar accumulation of NHE3 in the AP-1 cells, another fibroblast cell line. Interestingly, in Caco-2 cells and in the native renal epithelium, NHE3 has been recently shown to accumulate within a subapical intracytoplasmatic compartment (1, 9). Some evidence suggest that, at least in Caco-2 cells, this subapical compartment may correspond to the recycling endosomal compartment accumulating NHE3 in non-polarized AP-1 and PS120 cells (38), implicating some important similarities between the pathways of intracellular recycling of NHE3 in non-polarized mesenchymal cells and in polarized epithelial cells.

The answer to the third question formulated above is related to a more general question raised by our findings, and namely whether the processes of constitutive and bFGF-regulated recycling of NHE3 are controlled by common or separate signaling mechanisms. One recently emerging candidate for a common denominator for both processes is a family of PI 3-kinases. The most abundant product of the PI 3-K activity in mammalian cells is phosphatidylinositol (3)P, which is also believed to play an important role in vesicle trafficking (39). PI 3-K was shown to be involved in the regulation of constitutive recycling of GLUT4 in adipose cells (14), and of TIR in K562 cells (13). Recently, Kurashima and colleagues reported involvement of PI 3-K in the constitutive recycling of NHE3 in AP-1 fibroblasts (11). Our results complemented these findings by showing that NHE3-GFP is constitutively recycling also in PS120 cells, and that PI 3-K activity is required for the regulation of this process. Similarly to AP-1 cells, in PS120 cells inhibition of PI 3-K affected both endo- and exocytic arms of NHE3 recycling, although the effect on $k_{\text{in}}$ was much stronger than that on $k_{\text{ex}}$ (Table I). Similar effect of PI 3-K inhibitors on both arms of recycling was reported for GLUT4 and for TIR (13, 14). These data implicate that either PI 3-K separately controls exo- and endocytic arm of constitutive recycling of NHE3, or it regulates a step common for both pathways. The latter mechanism has been suggested for recycling of GLUT4, where PI 3-K was shown to inhibit homologous vesicle fusion, a process common for both arms of recycling (13, 41). Finally, wortmannin as well as LY294002 could theoretically exert diverse effects on recycling of NHE3 by simultaneous inhibition of kinases other than PI 3-K. However, we do not think this was the case. Although wortmannin has been shown to inhibit several kinases including PI 4-kinase (42), myosin light chain kinase, and protein kinase C (43), it is a quite selective PI 3-K inhibitor at the concentration used in this study. Moreover, effects similar to wortmannin were observed when using LY294002, which has the inhibitory mechanism different from wortmannin and, at 50 $\mu$M, does not affect protein kinases potentially involved in regulation of NHE3 activity (i.e. PI 4-kinase or protein kinase C) (44).

Although dependence of the constitutive recycling of NHE3 on the PI 3-K activity has been reported previously, this is the first report on the involvement of PI 3-K in the bFGF-stimulated up-regulation of the PM expression of the exchanger. Interestingly, our data also suggest participation of a mechanism independent from PI 3-K in this regulation. This conclusion is based on our observation that bFGF stimulated NHE3 activity and PM expression despite inhibition of PI 3-K with wortmannin or LY294002. However, the magnitude of this stimulation was only about 50% of that observed in intact cells, thus suggesting that approximately half of the stimulatory effect of bFGF did not depend on PI 3-K activity. Similar phenomenon of a partial independence from PI 3-K activity was also reported for insulin-stimulated activity of GLUT4 in adipose cells (14). On the other hand, lack of any effect of insulin on GLUT4 activity in wortmannin-pretreated adipose cells has also been reported (45). Moreover, although PI 3-K has been shown to be involved in the EGF-stimulated activity of NHE3 in the rabbit ileal epithelium and in Caco-2 cells, pretreatment of cells with wortmannin completely abolished the stimulatory effect of the growth factor (16). The reason for this discrepancy is not clear.

Conclusions regarding kinetics of recycling of NHE3-GFP presented in this communication need an additional comment, due to certain limitations of the method used in our studies. Calculation of $k_{\text{in}}$ was based on the dynamics of accumulation of vesicles colocalizing eGFP and FM 4–64 within the REC. Theoretically, vesicles containing only FM 4–64 and arriving into the REC could also generate colocalizing pixels, due to the close proximity of these vesicles and an abundance of pre-existing membranes containing eGFP. However, more than 90% of all endocytic vesicles emerging in the peripheral cytoplasm shortly after exposure of cells to FM 4–64 contained both FM 4–64 and eGFP, suggesting that influx of FM 4–64 into REC practically paralleled that of NHE3-GFP. In respect to the apparent $k_{\text{ex}}$ value, it should be stressed that it actually reflects the rate of disappearance of FM 4–64 fluorescence (colocalizing with eGFP) from the REC area. Our approach did not let us distinguish between exocytosis and other processes which could possibly result in a decrease in FM 4–64 fluorescence within the REC. These might include sorting of NHE3-GFP molecules away from FM 4–64 within REC, and a homotypic fusion of endocytic vesicles resulting in a significant dilution and subsequent fading of membrane-bound FM 4–64. However, at least two pieces of evidence strongly suggest that the observed decrease in FM 4–64 fluorescence most likely did reflect exit of FM 4–64/NHE3-eGFP vesicles from REC to the PM: (i) disappearance of FM 4–64 fluorescence from REC was associated with appearance of new, yellow-orange particles in the peripheral cytoplasm, suggesting trafficking of REC-derived exocytic vesicles toward the PM (Fig. 5) and (ii) the apparent value of $k_{\text{ex}}$ and first order kinetics of disappearance of FM 4–64 from REC closely resembled the rates of exit of TIR and bulk membrane from recycling endosomal compartment reported previously (46, 47), thus suggesting similar nature of both processes.

In conclusion, by studying regulation of the NHE3-eGFP fusion protein expressed in PS120 cells, we have demonstrated that bFGF stimulates the activity of NHE3 by increasing the steady-state expression of the exchanger at the PM. Moreover, we have shown that this effect results from bFGF-stimulated increase in the rate constant for exit of endocytic vesicles containing NHE3 from the juxtanuclear recycling endosomal compartment. We have also shown that at least two mechanisms mediate the effect of bFGF on the recycling of NHE3, and that
only one of those mechanisms depends on PI 3-K activity. Finally, we confirmed previous observations that PI 3-K is involved (at least in mesenchymal cells) in the regulation of constitutive recycling of NHE3. However, whereas PI 3-K was apparently involved in the regulation of both endo- and exocytic arms of the constitutive recycling of NHE3, only the exocytic arm of recycling was affected by PI 3-K inhibition during stimulation of the exchanger by bFGF.

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