Regulation of the Epithelial Brush Border Na\(^+\)/H\(^+\) Exchanger Isoform 3 Stably Expressed in Fibroblasts by Fibroblast Growth Factor and Phorbol Esters Is Not through Changes in Phosphorylation of the Exchanger*

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The epithelial brush border Na\(^+\)/H\(^+\) exchanger isoform 3 (NHE3) is regulated by growth factors and protein kinases. When stably expressed in PS120 fibroblasts, NHE3 is stimulated by serum and fibroblast growth factor (FGF) and inhibited by phorbol esters. To examine the role of phosphorylation of NHE3 in growth factor/protein kinase regulation, NHE3 was C-terminally tagged with an 11-amino acid epitope of the vesicular stomatitis virus glycoprotein (VSVG) and stably expressed in Na\(^+\)/H\(^+\) exchanger null PS120 fibroblasts (PS120/NHE3V). NHE3V was regulated by serum, FGF, and phorbol ester in a manner identical to wild type non-VSVG-tagged NHE3. Phosphorylation of NHE3V was evaluated via immunoprecipitation with anti-VSVG antibody after \textit{in vitro} labeling of PS120/NHE3V cells with \(^{32}\text{P}\)orthophosphate. NHE3V was phosphorylated under basal conditions. However, FGF and PMA, under conditions in which these agonists regulate NHE3, altered neither the amount of phosphorylation of NHE3V as analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis and autoradiography nor two-dimensional phosphopeptide maps of tryptic digests of NHE3V. In contrast, while changes in NHE3V phosphorylation were not observed with serum exposure by one-dimensional SDS-polyacrylamide gel electrophoresis, two-dimensional studies showed increases in two phosphopeptides. Under all these conditions, phosphoamino acid analysis showed that NHE3V was phosphorylated only on serine residues. By cell surface protein biotinylation studies under basal conditions, at least 27\% of the NHE3V was expressed on the cell surface. To further analyze the phosphorylation status of the surface and intracellular forms of NHE3V under basal conditions and determine whether the amount of phosphorylation of the surface form changes upon serum, FGF, and PMA regulation, the surface form of NHE3V was separated from intracellular form by biotinylation/avidin-agarose precipitation. Under basal conditions, both intracellular and surface forms of NHE3V were phosphorylated. However, the amount of phosphorylation of the surface form of NHE3V did not change upon stimulation by serum and FGF and inhibition by PMA based on one-dimensional SDS-polyacrylamide gel electrophoresis and autoradiography. Thus, we conclude that when expressed in PS120 cells, while NHE3 is a phosphoprotein under basal conditions, its regulation by FGF and PMA is not by changes in the phosphorylation of NHE3, while regulation by serum may involve changes in its phosphorylation. Regulation of NHE3 probably involves intermediate associated regulatory proteins. The function of basal phosphorylation of NHE3 is not known.

The mammalian Na\(^+\)/H\(^+\) exchanger (NHE)\(^1\) gene family is made up of plasma membrane transport proteins that are involved in the regulation of intracellular pH (pH\(_i\)), cell growth, cell volume, and transeellular Na\(^+\) absorption (1, 2). Five mammalian Na\(^+\)/H\(^+\) exchanger isoforms have been identified with different tissue distributions and functional properties (2, 3), and three of these have been expressed and characterized in detail (NHE1, NHE2, NHE3). By Northern blot analysis and immunocytochemistry, NHE1 is present in nearly all cell types and is localized to the basolateral surfaces of renal and intestinal polarized epithelial cells (4), whereas NHE2 and NHE3 are predominantly epithelial and are co-localized to the brush border of these cells (5).

NHE3 is stimulated by FGF, serum, and okadaic acid but inhibited by phorbol esters and by calmodulin, the latter under basal [Ca\(^{2+}\)] conditions (6–8). Unlike NHE1, the regulation of NHE3 is through changes in the maximum exchange rate (\(V_{\max}\)) rather than an alteration in \(K'(H^+)\) (9). At a protein level, NHE1 and NHE3 share 50–60\% identity in amino acid sequences, which are predominantly localized in the N-terminal transmembrane domain (9, 10). The cytoplasmic tail, which is the main area for regulatory function and contains multiple putative protein kinase consensus sites, however, is very different, with only 20–30\% amino acid identity (9, 10). Like NHE1, in the absence of ATP, basal Na\(^+\)/H\(^+\) exchange activity of NHE3 is greatly decreased and growth factor/protein kinase regulation ceases (6). NHE3 is,

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\(^1\) The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger; PAGE, polyacrylamide gel electrophoresis; FGF, fibroblast growth factor; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; VSVG, vesicular stomatitis virus glycoprotein; pH\(_i\), intracellular pH; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; CHP, calcineurin-homologous protein.
either directly or indirectly, regulated by phosphorylation. Okadaic acid, a phosphatase inhibitor, increases the \( V_{\text{max}} \) of NHE3, which implies a basal phosphorylated state of at least some proteins involved in NHE3 regulation. Conversely, genistein, a tyrosine kinase blocker, inhibits basal Na\(^+\)/H\(^+\) exchange activity, which suggests that tyrosine phosphorylation may be involved in stimulation of basal Na\(^+\)/H\(^+\) exchange (8). In addition, by performing C-terminal truncations of NHE3 and expressing the truncated cDNAs in PS120 cells, several separate subdomains of the cytoplasmic C terminus of NHE3 were identified, including areas responsible for regulation by calmodulin, phorbol ester, okadaic acid, FGF, and serum (8). NHE3 is phosphorylated under basal conditions, and this has been shown for NHE3 stably transfected in PS120 cells and in another fibroblast cell line, AP-1 cells (11). Both the PS120 cells and AP-1 cells are deficient in endogenous NHEs (12, 13). The amount of phosphorylation of NHE3 increases with cAMP inhibition of NHE3 in AP-1 cells (11). In the present study, we determine whether stimulation of NHE3 by FGF and inhibition of this protein by phorbol ester are associated with or are due to changes in phosphorylation of NHE3 stably expressed in PS120 cells. These regulators were compared with serum with its mixed growth factors. This study was also designed to separate the cell surface and intracellular forms of NHE3 and to determine whether changes in the rate of Na\(^+\)/H\(^+\) exchange by NHE3 as caused by protein kinases/growth factors are associated with changes in cell surface NHE3 phosphorylation.

### Experimental Procedures

**Materials**—[\(^{32}\)P]Orthophosphoric acid was from NEN Life Science Products. Fetal bovine serum was from Hyclone Corp. (Logan, UT), basic FGF was from Boehringer Mannheim, and the rest of the reagents were purchased from Sigma. The monoclonal (mouse) anti-VSVG antibodies were generous gifts from Drs. D. Ludwig and T. Kreis. The polyclonal anti-phosphotyrosine antibody was obtained from Zymed. NSS-SS-biotin and avidin-agarose were from Pierce. Protein A-Sepharose 6M beads were from Pharmacia Biotech Inc. Monoclonal anti-actin antibody (clone JLA20) was from Calbiochem.

**Construction and Stable Expression of Wild-type NHE3V cDNA**—To construct the VSVG epitope tag on the C terminus of NHE3 (NHE3V), we made use of an NHE3 truncation mutant, E3/S85, which had been VSVG-tagged on the C terminus. E3/S85 is a cDNA of NHE3 truncated from the C terminus at amino acid 585. Briefly, E3/S85 cDNA was obtained by cleaving the untagged E3/S85 cDNA from the pMAMneo vector with BamHI and XhoI. This BamHIXhoI cassette was subcloned into the 5'-end of the VSVG tag sequence in pBluescript (kindly provided by Dr. D. Ludwig). The XhoI site was retained at the boundary of the VSVG tag sequence and the E3/S85 cDNA. The tag sequence, when translated, contained an 8-amino acid spacer arm sequence (GGEGPFPQC) followed by the 11-amino acid VSVG epitope (YTDIEM-NRLGK). To obtain the E3pBluescript construct, a 0.9-kilobase pair NHE3 C terminus cDNA fragment (nucleotides 1536–2496, corresponding to amino acids 581–832) was obtained by polymerase chain reaction using the paired primers (5' primer, GCTGCCAGAAGTCTCGG; 3' primer, TTTCCTCAGATGGTGTGGACCTCGGG) where the 3' primer contained the NHE3 stop codon mutated into an XhoI site. This polymerase chain reaction fragment was then cloned into the BamHIXhoI (corresponding to amino acids 581–832) to replace the Stul-XhoI fragment in E3/S85VpBluescript, thus generating NHE3VpBluescript. The NHE3V cDNA was then subcloned into pMAMneo, generating NHE3VpPMAMneo.

PS120 cells were transfected with the NHE3VpPMAMneo construct using the calcium phosphate precipitation technique and then subjected to acid selection 3 days after the transfection. This procedure was repeated every 3–4 days over a period of 3 weeks. Geneticin (800 μg/ml) was present on initial splitting in the cell culture medium to further enhance selection pressure (5, 10).

### Measurement of Na\(^+\)/H\(^+\) Exchange—Cellular Na\(^+\)/H\(^+\) exchange activity in PS120/NHE3V cells was determined fluorometrically using an intracellular pH-sensitive dye BCECF with a fluorometer and a perfusion system with cells grown on glass coverslips and serum-starved overnight, as described previously (6–8). The effect of FGF (10 ng/ml) and PMA (1 μM) on NHE3 was studied by initial rates. The effect of 10% dialyzed FBS on NHE3V was studied by the initial rates and at steady-state pH, as described previously (6–8). In these studies, the rate of Na\(^+\)-dependent alkalization was obtained by calculating the first order derivative of the Na\(^+\)-dependent pH recovery curve. The hydrogen ion efflux rate (\( \mu \text{M} H^+/\text{s} \)) equivalent to the rate of Na\(^+\)/H\(^+\) exchange, was then determined by multiplying the rate of change in pH by the cellular buffering capacity at the corresponding pH. Scatter plots of H\(^+\) efflux rate versus intracellular H\(^+\) were constructed (6). Na\(^+\)/H\(^+\) exchange rate data were analyzed using a nonlinear regression data analysis program, ENZFITTER (Biosoft Corp.). In each experiment, control cells were studied at the same time in parallel with treated cells (FGF, 10 ng/ml, or PMA, 1 μM) to control for the variability in the basal exchange rate among cells from different cell passages and acid selection.

### In Vivo Labeling of Cells with \(^{32}\)P/Orthophosphate—Confluent PS120 or PS120/NHE3V cells grown in 10-cm dishes were washed twice with phosphate-free Dulbecco's modified Eagle's medium. Cells were then labeled in vivo for 4 h with 3 ml of phosphate-free Dulbecco's modified Eagle's medium containing 2.5 μCi of \(^{32}\)P/Orthophosphate. At the end of the incubation, cells were treated with 10% dialyzed FBS, PMA (1 μM), or FGF (10 ng/ml) for 5 h. It has previously been shown that under such conditions, these growth factors affect Na\(^+\)/H\(^+\) exchange by PS120/NHE3V cells (6–8). In the present study, we also tested whether these growth factors regulate PS120/NHE3V cells under the conditions used for phosphorylation. Cells were then washed with ice-cold phosphate-buffered saline at the end of the incubation period and processed immediately for immunoprecipitation.

### Immunoprecipitation—All procedures of immunoprecipitation were performed at 4 °C. The confluent layer of cells labeled with \(^{32}\)P/Orthophosphate as described above were scraped and resuspended in 500 μl of 50 mM Hepes/Tris, pH 7.4, 150 mM NaCl, 3 mM KCl, 25 mM sodium pyrophosphate, 5 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM p-aminonitrobenzil and 1 mM iodoacetamide) (IP buffer) (14–16). Cells were then treated by centrifugation for 10 min at 12,000 × g in an Eppendorf centrifuge and were resuspended in IP buffer containing 1% Triton X-100 (IPT buffer), sonicated for 20 s, and agitated on a rocking rotor at 4 °C for 30 min, followed by centrifugation at 12,000 × g for 30 min to remove insoluble cellular debris. The supernatants were first precleared with Protein A-Sepharose 6M beads by rocking for 1 h. The beads were spun down, and the supernatants were incubated overnight with the anti-VSVG monocular antibody, Protein A-Sepharose beads previously treated with PS120 cell extract solubilized by Triton X-100 (1%) were then added and allowed to rock for a further 2 h. The beads were then washed eight times with IPT buffer. Immunoprecipitated proteins were eluted by boiling in 70 μl of Laemmli sample buffer. For each sample, an aliquot of 50 μl was analyzed by electrophoresis on a 10% SDS-polyacrylamide gel, and autoradiography on a dried gel was performed.

The phosphoprotein corresponding to NHE3 was further characterized by two-dimensional tryptic phosphopeptide mapping and phosphoamino acid analysis.

### Two-dimensional Tryptic Phosphopeptide Mapping—The band corresponding to NHE3 was identified by autoradiography and prestained high molecular weight markers (Bio-Rad), excised, and washed (10% methanol, 5% glacial acetic acid and then washed with 50% methanol). The gel pieces were incubated with 0.3 mg/ml 1-tosylamide-2-phenyl-ethylichloromethyl ketone (TPCK)-treated trypsin in 0.4% NH4HCO3 at 37 °C overnight. The digested proteins were lyophilized and resuspended in 10 μl of H2O and spotted at the origin on thin layer cellulose plates together with 0.5 μl of each of the marker dyes, phenol red and basic fuchsin. Phosphopeptide fragments were separated in an acetic acid/pyridine/H2O (10:1:89) (pH 3.5, 500 V) in the first dimension until the marker dyes had travelled ~6 cm from the origin in opposite directions, and then by ascending chromatography in pyridine/butanol/ acetic acid/H2O (15:10:3:2) in the second dimension until the marker dyes had advanced to the edge of the thin layer plate. Analysis of the amount of phosphorylation of each phosphopeptide was by use of a
Phosphorylation of NHE3

RESULTS

Regulation of NHE3 by Serum, PMA, and FGF—We have previously shown that FBS and FGF stimulate, whereas PMA inhibits, NHE3 (6–8). Mechanistically, all growth factors regulate NHE3 by a $V_{\text{max}}$ change (6). To confirm that the VSVG epitope on NHE3 does not interfere with growth factor regulation of NHE3, we studied the effect of FBS, FGF, and PMA on NHE3 stably expressed in PS120 cells. As shown in Fig. 1A, when PS120/NHE3V cells, which were exposed to sodium medium (containing 130 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgSO$_4$, 1 mM NaPO$_4$, 2.5 mM glucose, 20 mM HEPES, pH 7.4) and had come to a steady-state pH, were then exposed to 10% FBS, an increase in pH $i$ occurred. This intracellular alkalization induced by serum could be blocked by 1 mM amiloride, confirming the activation of Na$^+$/H$^+$ exchange by NHE3V (decreased from 1796 ± 88 μM/s to 1086 ± 88 μM/s, a 40% decrease) in both cases, there is no change in K$'$ (H$^+$) or the Hill coefficient. Similarly, 10% FBS stimulated the initial rate of Na$^+$/H$^+$ exchange by NHE3V by respectively).

PhosphorImager and ImageQuant software, with a comparison made by normalization to spots felt not to change in phosphorylation in response to serum, PMA, and FGF, based on preliminary studies. Comparison was by paired $t$ tests.

Phosphoamino Acid Analysis—To determine the identity of the phosphorymo acids of NHE3, $^{32}$P-labeled protein was excised from acrylamide gels, treated with trypsin, and lyophilized as described above. The lyophilized protein was further hydrolyzed in 6 M HCl under nitrogen at 105 °C for 1 h. The acid-hydrolyzed peptides were rehydrolphized, resuspended in 10 μl of H$_2$O, and spotted on thin layer cellulose plates with 100 μg each of phosphoserine, phosphothreonine, and phosphotyrosine used as internal standards and 0.5 μl of phenol red marker also added at the origin. The plate was subjected to the first phase of electrophoresis in formic acid/acetiacid/H$_2$O (10:1:89) (pH 1.9, 500 V) until the phenol red had moved ~5 cm from the origin, and then electrophoresis was continued in the same direction until the marker had travelled another ~8 cm in another tank containing acetic acid/pyridine/H$_2$O (10:1:89) (pH 3.5, 500 V). The cellulose plates were dried, developed in 1% ninhydrin in acetone to identify the internal phosphoamino acid standards, and then subjected to autoradiography to reveal the $^{32}$P-labeled phosphoamino acids.

Cell Surface Biotinylation—Cell surface biotinylation was performed at 4 °C. PS120/NHE3V cells were grown to confluence in 10-cm Petri dishes. Cells were washed twice in phosphate-buffered saline (150 mM NaCl, 20 mM NaaHPO$_4$, pH 7.4) and once in borate buffer (54 mM NaCl, 10 mM boric acid, 7.2 mM KCl, 1.8 mM CaCl$_2$, pH 9) (17, 18). The surface plasma membrane proteins were then biotinylated by gently shaking the cells for 20 min with 3 ml of borate buffer containing 1.5 mg of NHS-SS-biotin (biotinylation solution). An additional 3 ml of the same biotinylation solution was then added, and the cells were rocked for an additional 20 min. The biotinylation solution was then discarded, and the cells were washed extensively with the quenching buffer (20 mM Tris and 120 mM NaCl, pH 7.4) to scavenge the unreacted biotin, and then the cells were washed twice with phosphate-buffered saline. Cells were then scraped and solubilized with 500 μl of IPT buffer and were then sonicated for 20 s and agitated on a rotating rocker at 4 °C for 30 min, followed by centrifugation at 12,000 × g for 30 min to remove insoluble cellular debris. The supernatant was then incubated with avidin-agarose to separate the biotinylated proteins from nonbiotinylated proteins by binding the former to avidin-agarose. Separation and analysis of the surface form from the intracellular form of NHE3V was performed as detailed in the figure legends.

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PhosphorImager and ImageQuant software, with a comparison made by normalization to spots felt not to change in phosphorylation in response to serum, PMA, and FGF, based on preliminary studies. Comparison was by paired $t$ tests.
Phosphorylation of NHE3V: Basal and Growth Factor/Protein Kinase-activated Conditions—Since NHE3 is regulated by serum, PMA, and FGF in the same way these growth factors/protein kinases regulate NHE3 and the anti-VSVG antibody allowed quantitative immunoprecipitation of NHE3V, we determined whether NHE3V is a phosphoprotein under basal conditions and whether serum, FGF, and PMA change the amount of its phosphorylation. PS120/NHE3V cells were labeled in vivo with [32P]orthophosphate, and NHE3V was immunoprecipitated with a polyclonal anti-VSVG antibody. The immunoprecipitated NHE3V was then analyzed by SDS-PAGE and autoradiography. As shown in Fig. 2A, under basal conditions, the rabbit polyclonal anti-VSVG antibody immunoprecipitated a single phosphoprotein of 85 kDa, which was recognized by the mouse monoclonal anti-VSVG antibody by Western blotting (Fig. 2B). This is the same size of the protein recognized on Western analysis by anti-NHE3 antibody (5). There was no detectable phosphoprotein immunoprecipitated by anti-VSVG antibody from untransfected PS120 cells (Fig. 2A). These results indicate that anti-VSVG antibodies specifically immunoprecipitate NHE3V, and NHE3V is an 85-kDa phosphoprotein under basal conditions.

In contrast to our original expectation, as shown in Fig. 2A, there was no change in the degree of total phosphorylation of NHE3V in response to a 5-min exposure to dialyzed FBS (10%), PMA (1 μM), or FGF (10 ng/ml). These findings were obtained in a series of five experiments, in which these regulators caused no consistent change in the amount of phosphorylation of NHE3. For instance, while serum apparently caused a small increase in phosphorylation of NHE3 in Fig. 2A, in other studies such as in Fig. 2B, this did not occur. Also, direct Cerenkov counting of the lyophilized immunoprecipitated digests gave similar counts of radioactivity among the control and samples treated with FBS, FGF, and PMA (data not shown).

To eliminate the possibility that any potential differences in phosphorylation could have been masked by unequal amounts of NHE3V being immunoprecipitated under various conditions, an aliquot of the immunoprecipitated NHE3V was separated by SDS-PAGE and transferred onto nitrocellulose. Then, sequentially on the same blots, autoradiography followed by Western analysis was performed to analyze the amount of NHE3V phosphorylation and protein, respectively, in the same specimen (Fig 2B). It was shown that the amount of NHE3V phosphorylation per amount of NHE3V protein was constant under basal, FBS-, FGF-, and PMA-regulated conditions. Thus, stimulation of NHE3 transport activity by FBS and FGF and its inhibition by PMA are not associated with detectable changes in total phosphorylation of the exchanger protein.

Phosphorylation of NHE3V: Two-dimensional Phosphopeptide Maps—While there is no change in the total amount of phosphorylation of NHE3V in response to FBS, FGF, and PMA, it was possible that there was an increase in phosphorylation of one site of NHE3V that was too small to change total NHE3 phosphorylation and/or a simultaneous decrease of another site, leading to no change in the total amount of NHE3V phosphorylation. It was also possible that there was a site that was dominant in the amount of phosphorylation and masked any changes in the amount of phosphorylation of other sites in response to growth factors/protein kinases. Therefore, we performed two-dimensional phosphopeptide mapping on immunoprecipitated NHE3V.

As shown in Fig. 3A, two-dimensional tryptic phosphopeptide mapping reproducibly revealed 14 phosphopeptides of NHE3 under basal conditions. However, the patterns of maps obtained in the basal state were indistinguishable from those obtained upon treatment with PMA (1 μM)-inhibited and FGF (10 ng/ml) and serum (10%)-stimulated conditions. PS120/NHE3V cells were labeled in vivo with [32P]orthophosphate, transport was altered under growth factor-regulated conditions, and then NHE3V was immunoprecipitated with the rabbit polyclonal anti-VSVG antibodies. The immunoprecipitated was then analyzed by SDS-PAGE and autoradiography, which showed that NHE3 is an 85-kDa phosphoprotein. PMA, FGF, and serum, which regulate NHE3V, did not change the amount of the phosphorylation on NHE3V compared with the control. PS120 cells were used as a negative control. B, to ensure that equal amounts of immunoprecipitate obtained from each condition were used for SDS-PAGE and autoradiography, PS120/NHE3V cells were labeled in vivo under basal and FGF-, PMA-, and serum-stimulated conditions as described in A, and the immunoprecipitated NHE3V was separated by SDS-PAGE and transferred onto nitrocellulose membrane, which was then exposed to obtain an autoradiograph (upper panel). After autoradiography, the same nitrocellulose membrane was used to analyze the amount of NHE3V protein by Western blotting using the mouse monoclonal anti-VSVG antibody, and the blot was detected by ECL (lower panel). This confirms that serum, FGF, and PMA do not change the amount of NHE3V phosphorylation and that similar amounts of immunoprecipitate were obtained under all conditions. This is a representative of five similar experiments. Molecular size markers are indicated on the left.
Phosphopeptide 12 as a percentage of phosphopeptide 3 phosphorylation was as follows: control, 45.68%; FBS, 135.35%, n = 3, p < 0.05.

Phosphoamino Acid Analysis—To determine the types of phosphoamino acids in NHE3V, the phosphorylated NHE3V immunoprecipitate was subjected to phosphoamino acid analysis as described under “Experimental Procedures.” Under basal conditions, the phosphoamino acids detected were entirely phosphoserine, with no phosphothreonine or phosphotyrosine identified (Fig. 4). Identical findings were obtained under FGF (10 ng/ml), PMA (1 μM) -exposed conditions. FGF stimulates and PMA inhibits NHE3V (Fig. 1 and Refs. 6 and 7). Simultaneous exposure of FGF (10 ng/ml) and PMA (1 μM) to PS120/NHE3V cells also revealed only phosphoserine but no phosphotyrosine and phosphothreonine in NHE3V immunoprecipitates (Fig. 4). The absence of tyrosine phosphorylation on NHE3V immunoprecipitate was also confirmed with a polyclonal antiphosphotyrosine antibody by Western blotting on basal, FGF-, PMA-, and FGF plus PMA-exposed conditions (data not shown).

Separation of Surface and Intracellular Forms of NHE3V: Phosphorylation of Surface NHE3V in Response to Serum and FGF Stimulation and PMA Inhibition—It is possible that in an overexpressing system, such as the expression of NHE3 in PS120 cells (7), not all expressed proteins are targeted to the plasma membranes. It is also not known whether the intracellular pools of NHE3 are phosphoproteins, although these pools of NHE3 are believed not to be involved in the exchange function of NHE3, at least under basal conditions. Therefore, we estimated the fraction of total NHE3 protein that is on the cell surface and determined whether the phosphorylation of cell surface NHE3 changed in response to growth factor/protein kinase regulation.

The surface and intracellular forms of NHE3V were separated by biotinylation of membrane surface proteins and subsequent affinity binding of biotinylated membrane surface proteins to avidin-agarose. Biotinylation was performed at 4 °C to restrict the biotin labeling to the cell surface proteins by minimizing internalization (17). After biotinylation, the cells were solubilized with IPT buffer, and the solubilized crude extract was incubated with avidin-agarose to which biotinylated surface proteins bound (avidin fraction), and the surface NHE3V was identified by Western blotting with anti-VSVG antibodies (Fig. 5, lane 2). Repeated incubation of the solubilized crude extract with avidin-agarose did not increase the recovery of the biotinylated NHE3V (data not shown). The crude extract after clearing with avidin-agarose represents the intracellular form of NHE3V and was analyzed by Western

**Fig. 3. Two-dimensional phosphopeptide mapping of NHE3V.** PS120/NHE3V cells were labeled in vivo and then treated under basal, FGF-, PMA-, and serum-exposed conditions as described in the legend to Fig. 2. NHE3V was then immunoprecipitated with the rabbit polyclonal anti-VSVG antibody. The immunoprecipitated protein was separated by SDS-PAGE. The polyacrylamide gel was dried and exposed for autoradiography. The phosphoprotein band (NHE3V immunoprecipitate) was sliced out and digested with trypsin (0.3 mg/ml, TPCK-treated). The tryptic phosphopeptides were analyzed in the first dimension by thin layer electrophoresis and in the second dimension by thin layer ascending chromatography. The two-dimensional phosphopeptide map was analyzed by autoradiography. A, two-dimensional tryptic phosphopeptide mapping reproducibly revealed 14 phosphopeptides in the control NHE3V phosphopeptide map. B, identical two-dimensional phosphopeptide maps of NHE3V were obtained under the control basal condition and FGF- and PMA-regulated conditions. Under FBS conditions, a relative increase in size of phosphopeptides 9 and 12 was reproducibly seen when compared with the control. Shown is a representative of five similar studies.

**Fig. 4. Phosphoamino acid analysis of NHE3V.** Phosphoamino acid analysis was performed on 32P-labeled NHE3V immunoprecipitate obtained under basal and FGF (10 ng/ml)-, PMA (1 μM)-, and FGF (10 ng/ml) plus PMA (1 μM)-regulated conditions as described under “Experimental Procedures.” The results showed that the phosphoamino acids detected were entirely phosphoserine with no phosphothreonine or phosphotyrosine under basal, FGF-, PMA-, and FGF plus PMA-treated conditions. This is a representative of two similar experiments.
FIG. 5. Separation of cell surface and intracellular forms of NHE3. PS120/NHE3V cells were biotinylated with NHS-SS-biotin at 4°C. After biotinylation, the cells were solubilized with IPT buffer, and a 1/5 of the total mixture (by volume) of the crude solubilized extract was analyzed by Western blotting using anti-VSVG antibody (lane 3, total NHE3V). The biotinylated surface NHE3V in the crude solubilized extract was then adsorbed onto avidin-agarose, and a 1/5 of the total mixture (by volume) of this fraction (lane 2, surface) was analyzed by Western blotting, to keep the amount of samples loaded into each lane the same for quantitation. The crude extract after clearing with avidin-agarose represents the intracellular form and a 1/5 of the total mixture (by volume) of this fraction was analyzed by Western blotting (lane 1) for quantitative comparison with total NHE3V (lane 3) and the surface form of NHE3V (lane 2). Quantitation by densitometry (ImageQuant software) revealed that ~27% of the 85-kDa NHE3V protein is expressed on the cell surface (compare lanes 2 and 3). Shown is a representative result of three experiments with similar results, with control surface NHE3V being 27 ± 8% of total NHE3V.

We next determined whether both the surface and intracellular forms of NHE3V are phosphoproteins. PS120/NHE3V cells were labeled in vivo with [32P]orthophosphate, and the surface plasma membrane proteins were biotinylated with NHS-SS-biotin as described under "Experimental Procedures." The cells were then solubilized with IPT buffer. To obtain the intracellular form of NHE3V, the solubilized crude cell extract was first incubated with 100 μl of avidin-agarose to remove biotinylated proteins. Remaining nonbiotinylated proteins were then immunoprecipitated with anti-VSVG antibody to recover the nonbiotinylated [32P]NHE3V. In parallel experiments to obtain the surface form of NHE3V, total [32P]NHE3V was immunoprecipitated from the solubilized crude cell extract with anti-VSVG antibody. The [32P]NHE3V was then eluted from the antigen-antibody complex with 100 μl of 10 mM Tris, 1% SDS. The eluted [32P]NHE3V was then diluted with 1 ml of IPT buffer. The biotinylated surface form of [32P]NHE3V was then recovered by incubating with avidin-agarose. Both forms of NHE3V were analyzed by SDS-PAGE and transferred onto nitrocellulose membranes, which were exposed to obtain an autoradiograph (upper panel). After autoradiography, the same nitrocellulose membrane was used to analyze the amount of NHE3V protein by Western blotting (lower panel). The surface plasma membrane proteins were then biotinylated. Total [32P]NHE3V was immunoprecipitated, followed by elution of the immunoprecipitated protein from the Protein A-Sepharose beads and re-affinity purification of the surface [32P]NHE3V by incubating with avidin-agarose. As shown in Fig. 6, there was no change in the amount of total phosphorylation of surface NHE3V in response to stimulation by serum and FGF and inhibition by PMA (1 μM) for 10 min. The surface plasma membrane proteins were then biotinylated. Total [32P]NHE3V was immunoprecipitated, followed by elution of the immunoprecipitated protein from the Protein A-Sepharose beads and re-affinity purification of the surface [32P]NHE3V by incubating with avidin-agarose. As shown in Fig. 7, there was no change in the amount of total phosphorylation of surface NHE3V in response to stimulation by serum and FGF and inhibition by PMA (1 μM) when normalized to the amount of surface NHE3V as indicated below by Western blotting (lower panel).

DISCUSSION

In the present studies, we showed that the epithelial isoform Na+/H+ exchanger, NHE3, exists as a phosphoprotein under basal conditions. Regulation of the transport rate of NHE3 by FGF, FBS, and phorbol esters is not, however, accompanied by a simultaneous change in the degree of total phosphorylation of NHE3, compared with the basal state. Further, two-dimen-
FIG. 7. Phosphorylation of the surface form of NHE3V. PS120/NHE3V cells were labeled in vivo with $^{32}$P-orthophosphate. At the end of incubation, cells were exposed to serum, FGF, and PMA as described in the legend to Fig. 2 and under “Experimental Procedures.” The surface form of NHE3 was then obtained under the control and growth factor-regulated conditions as described in Fig. 6. The upper panel is an autoradiogram showing that serum, FGF, and PMA, which regulate NHE3, do not change the amount of the phosphorylation of surface NHE3 compared with the control. The lower panel is Western blotting confirming that similar amounts of the surface form of NHE3V were obtained under all conditions.

Phosphorylation of NHE3

There are alternate interpretations for failure to observe changes in phosphorylation of NHE3 in parallel with FGF and phorbol ester activity. In contrast, FBS increased phosphorylation of two phosphopeptides based on two-dimensional tryptic phosphopeptide maps. The magnitude of the change in phosphorylation of NHE3 phosphopeptides 9 and 12 in response to FBS (2–3-fold) is similar to that reported in regulation of other transport proteins (19, 20). These results suggest that, as for NHE1, NHE3 is regulated by mechanisms independent of phosphorylation of the exchanger (FGF, phorbol ester) as well as mechanisms that may be dependent on changes in phosphorylation of NHE3 (FBS). However, we are aware that we have not established that the changes in phosphorylation of NHE3 are involved in its regulation by FBS. This is especially important to note, since FBS causes the largest magnitude change in NHE3 stimulation among agents studied, although it is not known which growth factor is responsible.

There are alternate interpretations for failure to observe changes in total phosphorylation of NHE3 in parallel with FGF and phorbol ester regulation of NHE3 transport activity. For instance, the C-terminal VSVG epitope tag could have interfered with FGF/phorbol ester regulation of NHE3 and therefore of phosphorylation. This was shown not to be the case based on the FBS/phosphorylation studies as well as on the fact that PS120/NHE3V responded similarly to non-epitope-tagged NHE3 with stimulation by FGF and inhibition by PMA (Fig. 1). These results do exclude the possibility that the lack of change in total phosphorylation of NHE3 by FGF and phorbol esters is due to the selective dephosphorylation of the sites phosphorylated under basal conditions with concomitant phosphorylation of other sites. Furthermore, as a parallel positive control experiment using identical experimental procedures, we confirmed that NHE1 is a phosphoprotein and that its amount of phosphorylation increases with response to serum stimulation of NHE1 (data not shown), as reported (10, 11).

Similarly to the results shown here with NHE3, regulation of NHE1 occurs by phosphorylation-dependent and -independent mechanisms. NHE1 is not only a phosphorylated protein under basal conditions, but also growth factors/protein kinases activated NHE1 and increased its phosphorylation in parallel (14–16). While there are two major signaling pathways of NHE1 expressed in fibroblasts (thrombin/PI turnover; epidermal growth factor/tyrosine phosphorylation), both pathways increased phosphorylation of the same set of NHE1 phosphopeptides, indicating action via a common kinase intermediate (16). It was recently shown that the location of the NHE1 phosphorylation sites was C-terminal of amino acid 635, in the cytoplasmic tail of NHE1, which contains 8 serine residues (16). However, growth factors/kinases were still able to cause 50% activation of an NHE1 truncation mutant that lacks the entire phosphorylation domain. This suggests that 50% of the growth factor/kinase regulation of NHE1 is phosphorylation-independent and may involve associated regulatory proteins (16).

The concept of regulation by associated regulatory proteins was further supported by the observation that osmotic regulation of NHE1 occurs without changes in NHE1 phosphorylation (13). In fact, three NHE1-associated regulatory proteins have been identified. Wakabayashi showed that calmodulin (Cam) binds to two separate domains with different affinities on the cytoplasmic C terminus of NHE1. The high affinity binding site normally inhibits NHE1 activity, and in the presence of Ca$^{2+}$, binding of calmodulin to the high affinity site leads to stimulation of NHE1 activity (22–24). Grinstein and co-workers (25) identified an NHE1-associated protein, p24, which co-immunoprecipitates with NHE1. This protein is not constitutively phosphorylated, nor could phosphorylation be induced by serum or phorbol ester.

Binding of this protein to NHE1 is Ca$^{2+}$-independent. However, the identity of this regulatory associated protein is not yet known. Independently, Lin and Barber (26) used an interaction cloning approach to identify NHE1 regulatory proteins by using a glutathione S-transferase fusion protein of the NHE1 C-terminal cytoplasmic domain as a hybridization probe to screen an expression library. They identified a calcineurin B- and calmodulin-homologous protein, which they called calcineurin-homologous protein (CHP). CHP binds to the “maintenance domain” of NHE1 and is a phosphoprotein. Interestingly, serum stimulates NHE1 activity and increases NHE1 phosphorylation. Expression of CHP inhibits serum-stimulated NHE1 activity, while the amount of phosphorylation of CHP decreases in response to serum. Therefore, it is suggested that the phosphorylation state of the CHP is important for regulation of NHE1 and that it has an inhibitory effect.

A strong case can be made that some regulation of NHE3 is via associated regulatory proteins, with the associated proteins probably regulated directly or indirectly by phosphorylation. The clearest example is calcmodulin (CaM). CaM binds and regulates NHE1 and also regulates and binds NHE3 (8, 24). Inhibiting CaM stimulates NHE3, with the CaM acting by both CaM kinase II-dependent and -independent mechanisms (11). CaM binds to the C terminus of NHE3 and does so in the same area that is required for its effect on Na$^{+}/H^+$ exchange (24).

Further evidence to support a role for associated regulatory proteins in regulating NHE activity comes from studies of the cell specificity of NHE regulation. Regulation by protein kinases of NHE3 stably expressed in PS120 cells and in the human colon cancer cell line, Caco-2 cells, is remarkably similar and also, with one exception, is the same as the regulation of NHE3 expressed endogenously on the apical membrane of the rabbit small intestine and rat colon. The major difference is that cAMP inhibits intestinal brush border NaCl absorption and brush border Na$^{+}/H^+$ exchange, at least in some studies, while cAMP has no effect on NHE3 in PS120 cells and on the apical surface of Caco-2 cells (2, 6). In contrast, Moe et al. showed that when rat NHE3 is stably expressed in AP-1 cells, cAMP inhibits NHE3 along with causing an increase in NHE3 phosphorylation as determined by immunoprecipitation of NHE3 after in vivo phosphorylation (11). These results show that protein kinase A inhibits NHE3 stably expressed in AP-1 cells along with increasing NHE3 phosphorylation. To date, as with the FBS-induced increase in NHE3 phosphorylation re-

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2 C.-M. Tse, R., Kambadur, M. Zizak, S. Nath, and M. Donowitz, unpublished observation.
ported here, there has been no demonstration that stimulation by cAMP of NHE3 phosphorylation in AP-1 cells is what inhibits NHE3 activity.

The simplest explanation for the differences in cell-specific NHE regulation is that there are cell-specific differences in associated regulatory proteins, since, for instance, cAMP in NHE regulation is that there are cell-specific differences in protein were recently cloned by Weinman et al. (27) and Yun et al. (28), respectively. These proteins are suggested as mediating cAMP inhibition of NHE3 in brush border membranes and AP1 cells but not in PS120 cells. Recently, it was shown that co-expression of Na+/H+ exchanger regulatory factor or NHE3 kinase A regulatory protein with NHE3 in PS120 cells reconstitutes cAMP inhibition of NHE3 (28).

The function of basal NHE3 serine phosphorylation is not known. Multiple phosphorylation sites of NHE3 exist, since at least 14 phosphopeptides were generated by trypsin digestion when studied by two-dimensional phosphopeptide mapping (Fig. 3A). Possibilities for the function of basal or FBS-stimulated phosphorylation in NHE3 include plasma membrane targeting or removal from the plasma membrane. Recently, based on preliminary studies, we suggested that vesicle membrane shuffling is involved in regulation of NHE3 in rat kidney (29). Also unknown is whether basal NHE3 phosphorylation is required for associated protein regulation of NHE3.

In conclusion, NHE3 stably transfected in PS120 fibroblasts is a phosphoprotein under basal and growth factor/kinase-regulated conditions. Moreover, similar to results with NHE1, growth factor/kinase regulation of NHE3 involves both NHE3 phosphorylation-independent (FGF, phorbol ester) and potentially phosphorylation-dependent (FBS) mechanisms when NHE3 is studied in PS120 cells, the same cell in which NHE1 was studied. The difference between NHE1 and NHE3 regulation is that, for NHE3, certain regulators act independently of changes in phosphorylation (FGF, phorbol esters), while these same regulators of NHE1 act by both phosphorylation-dependent and phosphorylation-independent components. Our results suggest that NHE3 stably transfected in PS120 fibroblasts is regulated at least by FGF and phorbol esters via associated regulatory proteins. Unknown is whether NHE3 is regulated by both phosphorylation-dependent and -independent mechanisms in epithelial cells of intestine and kidney, where NHE3 is localized to the brush border membranes.

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