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cAMP-induced Phosphorylation and Inhibition of Na^+/H^+ Exchanger 3 (NHE3) Are Dependent on the Presence but Not the Phosphorylation of NHE Regulatory Factor*

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The members of the regulatory factor (RF) gene family, Na^+/H^+ exchanger (NHE)-RF and NHE3 kinase A regulatory factor (E3KARP) are necessary for cAMP to inhibit the epithelial brush border NHE isoform 3 (NHE3). The mechanism of their action was studied using PS120 fibroblasts stably transfected with rabbit NHE3 and wild type rabbit NHE-RF or wild type human E3KARP. 8-Bromo-cAMP (8-Br-cAMP) had no effect on Na^+/H^+ exchange activity in cells expressing NHE3 alone. In contrast, in cells co-expressing NHE-RF, 8-Br-cAMP inhibited NHE3 by 39%. *In vivo* phosphorylation of NHE3 demonstrated that cAMP increased phosphorylation in two chymotrypsin-generated phosphopeptides of NHE3 in cells containing NHE-RF or E3KARP but not in cells lacking these proteins. The requirement for phosphorylation of NHE-RF in this cAMP-induced inhibition of NHE3 was examined by studying a mutant NHE-RF in which serines 287, 289, and 290 were mutated to alanines. Wild type NHE-RF was a phosphorylated protein under basal conditions, but treatment with 8-Br-cAMP did not alter its phosphorylation. Mutant NHE-RF was not phosphorylated either under basal conditions or after 8-Br-cAMP. 8-Br-cAMP inhibited NHE3 similarly in PS120/NHE3 cells containing wild type or mutant NHE-RF. NHE-RF and NHE3 co-precipitated and did so similarly with and without cAMP. Mutant NHE-RF also similarly immunoprecipitated NHE3 in the presence and absence of 8-Br-cAMP. This study shows that members of the regulatory factor gene family, NHE-RF and E3KARP, are necessary for cAMP inhibition of NHE3 by allowing NHE3 to be phosphorylated. This inhibition is not dependent on the phosphorylation of NHE-RF.

It is now established that cAMP-dependent inhibition of NHE3,[1] the epithelial brush border isoform Na^+/H^+ exchanger, requires the presence of associated regulatory proteins of the regulatory factor (RF) gene family (1–8). There are two identified members of this family, Na^+/H^+ exchanger regulatory factor (NHE-RF) and NHE3 kinase A regulatory protein (E3KARP) (1). Recent reports have suggested a model whereby NHE-RF, in association with PKA II and ezrin, functions as a signaling complex to regulate NHE3 activity (2, 9–12). Multiple aspects of this model have not been explicitly studied, although an increase in phosphorylation of NHE3 is necessary for the cAMP inhibition (4). The present experiments use PS120 fibroblasts stably expressing vesicular stomatitis virus glycoprotein (VSVG)-tagged NHE3 and either native NHE-RF or E3KARP or a mutant form of NHE-RF to study two phosphorylation-related aspects of the proposed signaling complex model through which cAMP inhibits NHE3. First, the mechanism by which cAMP regulates NHE3 activity was examined. Specifically, the question of whether NHE-RF or E3KARP is necessary for PKA to phosphorylate NHE3 was addressed. Second, although NHE-RF was originally isolated and characterized as a PKA substrate (7), recent *in vivo* experiments indicate that NHE-RF exists as a phosphoprotein in unstimulated human embryonic kidney (HEK293) cells and OK cells but that treatment of these cells with cAMP increased the phosphorylation of NHE-RF minimally or not at all (5, 7, 8). These prior *in vivo* studies, however, did not specifically correlate the relation between the effect of cAMP on NHE3 transporter activity and the phosphorylation state of NHE-RF. Accordingly, the functional and biochemical properties of a nonphosphorylated mutant NHE-RF containing serine to alanine mutations of residues 287, 289, and 290 (NHE-RF/S287A/S289A/S290A) was examined. The results confirmed that in PS120 cells expressing NHE3, there is an absolute requirement for the presence of NHE-RF or E3KARP for cAMP to inhibit transporter activity. These studies showed that NHE-RF or E3KARP is required for PKA-mediated phosphorylation of NHE3. On the other hand, phosphorylation of NHE-RF is not required for it to function as a co-factor in cAMP-mediated inhibition of NHE3.

MATERIALS AND METHODS

Cell Culture Models—Studies were performed using PS120/NHE3V fibroblasts, which lack endogenous NHE-RF or E3KARP (1). These are

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[1] The abbreviations used are: NHE3, Na^+/H^+ exchanger isoform 3; NHE-RF, Na^+/H^+ exchanger regulatory factor; E3KARP, NHE3 kinase A regulatory factor; RF, regulatory factor; PKA, protein kinase A; OK, opossum kidney; 8-Br-cAMP, 8-bromo-cAMP; VSVG, vesicular stomatitis virus glycoprotein; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

PS120 cells stably transfected with rabbit NHE3 tagged at its C terminus with an epitope derived from the VSVG, as described previously (13). Wild type and mutated NHE-RF and wild type E3KARP cDNAs were cloned into the pECE vector. PS120/NHE3V fibroblasts were co-transfected using Lipofectin (Life Technologies, Inc.) with the pECE/NHE-RF or E3KARP constructs and pPlo2 to permit selection by hygromycin (1). Cells resistant to 600 unit/ml hygromycin were selected through eight passages prior to the study. Transfected PS120 fibroblasts were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml).

The mutant form of NHE-RF was made using the MORPH™ mutagenesis kit (5 Prime → 3 Prime, Inc., Boulder, CO), and the mutation was confirmed by double-stranded sequencing (8). Serine residues 287, 289, and 290 were mutated to alanine residues using an oligonucleotide of the following sequence: 5′-GCTGGTGTCAGCGGCGGCGGCTCT-TGC-3′. Reverse transcription polymerase chain reaction was used to confirm successful transfections.

Na^+/H^+ Exchange—Na^+/H^+ exchange activity was determined in cells seeded on glass coverslips using the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5–6-carboxyfluorescein, NH_4Cl prepulse, and a computerized fluorometer, as described (2, 14). The cells were serum-deprived for 12–20 h prior to study. The NH_4Cl pulse was targeted to achieve an initial pH_i of 6.0, and only cells with initial pH_i values between 6.0 and 6.2 were included for analysis. Na^+/H^+ exchange activity, expressed as ΔpH_i/min, was calculated from the slope of the initial 10–15 s of sodium-dependent pH_i recovery. Over this time period, the relation between pH_i and time approximated a linear function. When studied, cells were pretreated with 100 μM 8-Br-cAMP during the final 15 min of the dye loading and continuously during the perfusion. At the end of each experiment, the cells were equilibrated in pH clamp media containing 20 mM HEPES, 20 mM MES, 110 mM KCl, 14 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM TMA, 25 mM glucose, and 10 mM nigericin at pH 6.1 and 7.2. All measurements in control and experimental cells were made on cells from the same passage and assayed on the same day.

In Vivo Phosphorylation of NHE3—To determine the *in vivo* phosphorylation of NHE3, cells were washed with phosphate-free Dulbecco's modified Eagle's medium and labeled *in vivo* for 4 h using the same medium containing 2.5 mCi of [^{32}P]orthophosphate. At the end of the incubation, half of the cells were treated with 100 μM 8-Br-cAMP for 15 min. Control and cAMP-treated cells were scraped and resuspended in 500 μl of a solution containing 60 mM HEPES/Tris, pH 7.4, 150 mM NaCl, 3 mM KCl, 25 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM phenanthroline, and 1 mM iodoacetamide) (IP buffer). Cells were collected by centrifugation for 10 min at 12,000 × *g* in an Eppendorf centrifuge and resuspended in IP buffer containing 1% Triton X-100 (IPT buffer), lysed by being drawn through a 23 gauge needle, and agitated on a rotating rocker at 4 °C for 30 min, followed by centrifugation at 12,000 × *g* for 30 min. The supernatants were first precleared with protein A-Sepharose 6M beads by rocking for 1 h. The beads were then spun down, and the supernatants were incubated overnight with 5 μl of anti-VSVG polyclonal antibody. Protein A-Sepharose beads previously treated with PS120 cell extract solubilized by 1% Triton X-100 were then added and allowed to rock for an additional 2 h. The beads were eluted by boiling in 70 μl of Laemmli SDS sample buffer. The phosphoprotein corresponding to NHE3 was identified on 10% SDS-polyacrylamide gels and autoradiography. For two-dimensional mapping, NHE3 was excised from the gels and washed in 10% methanol and 5% glacial acetic acid, followed by a wash in 50% methanol. The gel pieces were incubated with 100 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated chymotrypsin in 0.4 NH_4HCO_3 at 37 °C overnight, followed by a second, 8-h chymotrypsin digestion. The digested peptides were separated on thin layer chromatography plates, as described (5).

In Vitro Back-phosphorylation of NHE3—To extend these experiments, an *in vitro* "back-phosphorylation" assay was employed to assess PKA-mediated phosphorylation of NHE3. The rationale for this approach is based on the evidence that PKA phosphorylates a specific serine residue(s) in the C terminus of NHE3 (4). These sites should be available *in vitro* to be phosphorylated in NHE3 immunoprecipitated from untreated cells but not in cells treated with cAMP, in which they are occupied by an unlabeled phosphate residue. PS120 cells expressing VSVG-tagged NHE3 alone or in the presence of native or mutant NHE-RF were studied under basal conditions or after treatment of the cells with 100 μM 8-Br-cAMP for 15 min. The immunoprecipitation procedures were performed as indicated above using protein A-Sepharose beads. Samples were eluted with 100 μl of 30 mM glycine HCl, pH 2.8, and immediately neutralized with the addition of 10 μl of 1 M Tris, pH 11. Samples were phosphorylated at pH 7.4 for 10 min at 30 °C in the presence of 21 mM glycine, 100 mM Tris, pH 7.4, 50 μM $MgCl_2$, 180 units of the catalytic subunit of PKA (Promega), and 50 μCi of [γ-^{32}P]ATP.

The reaction was terminated by boiling in Laemmli buffer and run on 10% SDS-PAGE gel. The proteins were transferred to nitrocellulose, and the phosphoproteins were visualized by autoradiography. After autoradiography, Western immunoblotting using polyclonal anti-VSVG antibodies was performed to assess sample loading of the gels, and the immune complexes were detected by ECL (Amersham Pharmacia Biotech). The autoradiographs and Western immunoblots were quantitated using laser densitometry with ImageQuant software.

In Vivo Phosphorylation of NHE-RF—Methods similar to those described above were employed to determine the *in vivo* phosphorylation of NHE-RF. *In vivo* phosphorylated NHE-RF was immunoprecipitated from [^{32}P]orthophospate-labeled cells that had not been treated or had been treated with 100 μM 8-Br-cAMP, using a polyclonal antibody to recombinant full-length NHE-RF, which we had previously described (8). Antibody was conjugated to protein A-Sepharose beads using cyanogen bromide (8). The beads were then incubated overnight with the ^{32}P-labeled cell lysates and recovered by centrifugation. The beads were washed three times in lysis buffer, after which 100 ml of SDS-sample buffer was added, and the samples were heated to 85 °C for 10 min. The proteins were resolved on 6% polyacrylamide slab gels, which separated NHE-RF from IgG.

Co-immunoprecipitation—Co-immunoprecipitation experiments were performed using cell lysates from PS120 cells expressing NHE3V and either wild type or mutant NHE-RF in the absence or presence of 8-Br-cAMP. The lysates were split, and proteins were immunoprecipitated either using an anti-VSVG antibody or the anti-NHE-RF antibody. The individual antibodies were conjugated to protein A-Sepharose beads using cyanogen bromide. NHE3 and NHE-RF were resolved on 10 and 6% polyacrylamide gels, respectively.

Representative autoradiographs and Western immunoblots are shown. Statistical analysis of the Na^+/H^+ exchange transport rates and phosphorylation of specific phosphopeptides between control and experimental samples was performed using Student's *t* test for paired data.

RESULTS

cAMP Inhibition and Phosphorylation of NHE3 Require NHE-RF—PS120 cells transfected with NHE3V and rabbit NHE-RF were used to correlate the physiologic effect of cAMP to inhibit Na^+/H^+ exchange with the phosphorylation state of NHE3. Western immunoblot analysis on whole cell lysates using anti-VSVG antibody and a polyclonal antibody to full-length recombinant NHE-RF demonstrated that expression of NHE3V was similar in all cell lines and that expression of NHE-RF was approximately equal in the cell lines expressing it (data not shown). Na^+/H^+ exchange activity is expressed as sodium-dependent pH_i recovery following acidification of the cells. The rate of recovery was calculated in a restricted range of pH_i and over a time course that approximated an initial rate. The results are summarized in Table I. In PS120/NHE3V cells not co-transfected with NHE-RF, cAMP did not significantly inhibit NHE3. In contrast, cAMP inhibited NHE3 in cells transfected with wild type rabbit NHE-RF, the pH_i recovery decreasing from a rate of 1.78 ± 0.17 ΔpH_i/min in the absence of cAMP to 1.09 ± 0.17 in the presence of cAMP ($n = 6$, $p < 0.01$).

To determine the effect of 8-Br-cAMP on NHE3 phosphorylation, [^{32}P]orthophosphate loaded cells were exposed to 100 μM 8-Br-cAMP for 15 min. NHE3 phosphorylation was not significantly different in the absence or presence of 8-Br-cAMP incubation in cells lacking NHE-RF and E3KARP or in the cells with either NHE-RF or E3KARP based on one-dimensional SDS-PAGE (data not shown).

Fig. 1*A* shows the two-dimensional chymotrypsin digestion phosphopeptide maps of NHE3V. In control PS120/NHE3V cells, phosphorylation of NHE3 produced a pattern with five well defined phosphopeptides (Fig. 1*A, 1–5*). cAMP did not alter

TABLE I

The effect of cAMP on Na^+/H^+ exchanger activity in PS120/NHE3V cells

Summary of the effect of 100 μM 8-Br-cAMP on Na^+/H^+ exchange activity as determined by the initial rate of sodium-dependent pH_i recovery using 2′,7′-bis(carboxyethyl)5–6-carboxyfluorescein fluorescence. PS120 cells stably expressing rabbit NHE3 were co-transfected with either wild type rabbit NHE-RF or rabbit NHE-RF with serine to alanine mutations at residues 287, 289, and 290. Results are reported as ΔpH_i/min and expressed as means ± S.E. *n* refers to number of separate experiments.

NHE-RF	Control	cAMP	Inhibition
			%
None ($n = 6$)	2.22 ± 0.30	2.10 ± 0.20	2.5 ± 5.6
Wild type ($n = 6$)	1.78 ± 0.17	1.09 ± 0.17[a]	39.2 ± 6.2
Mutant S287A/S289A/S290A ($n = 6$)	1.82 ± 0.11	1.12 ± 0.03[a]	37.0 ± 4.8

[a] $P < 0.01$ (control *vs.* cAMP).

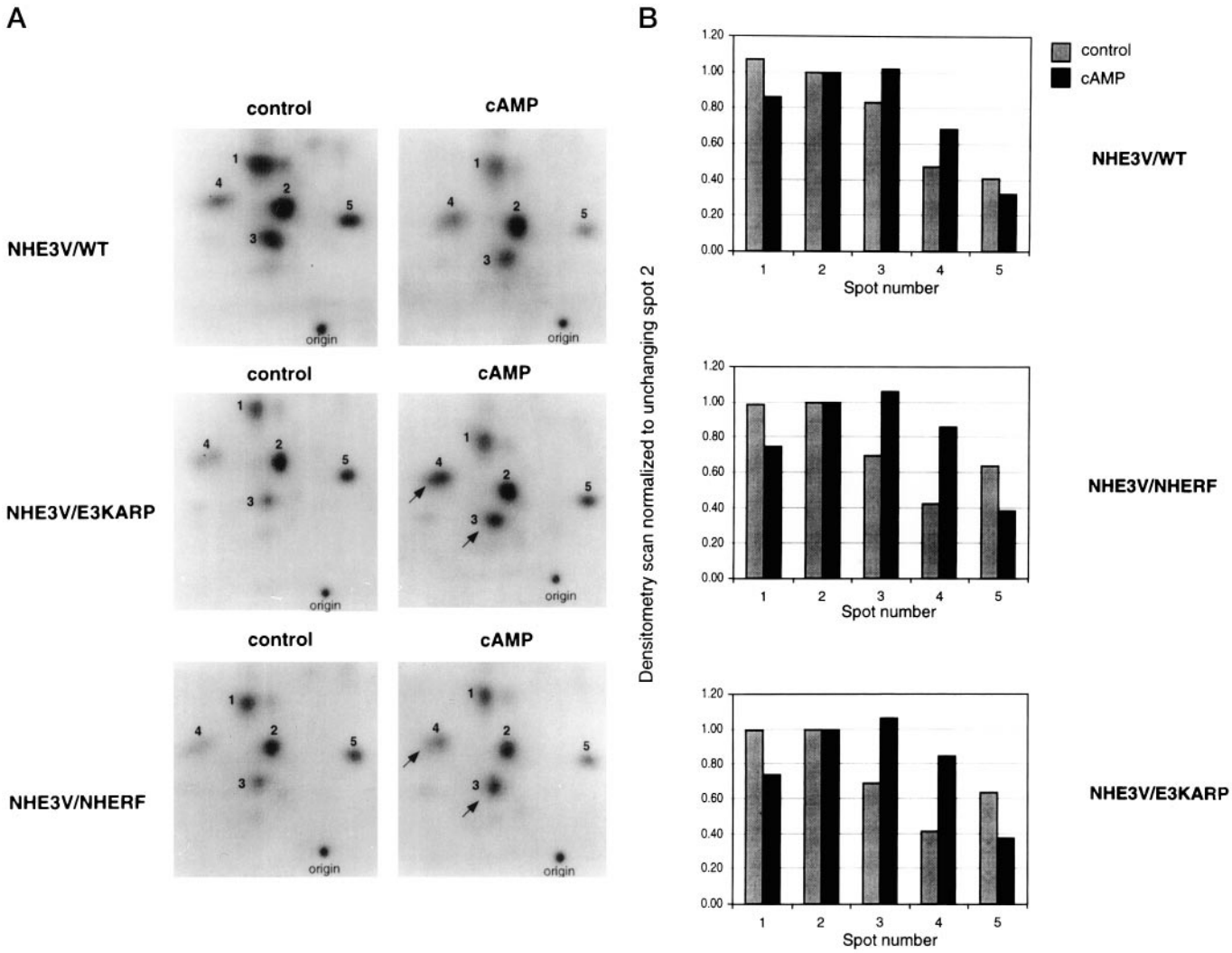

FIG. 1. **NHE-RF and E3KARP are necessary for 8-Br-cAMP-induced phosphorylation of NHE3.** *A,* two-dimensional phosphopeptide map of *in vivo* phosphorylated NHE3V in the absence and presence of 8-Br-cAMP. Control PS120/NHE3V and PS120/NHE3V cells stably transfected with E3KARP or NHE-RF were incubated with [^{32}P]orthophosphate for 4 h and treated with 100 μM 8-Br-cAMP for 15 min. NHE3V was immunoprecipitated and treated with chymotrypsin, as described under "Materials and Methods." The peptides were separated by electrophoresis and thin layer chromatography and visualized by autoradiography. A representative experiment is shown; similar results were found in a second identical experiment. *B,* quantification of the signal from individual phosphopeptides of chymotrypsin-digested NHE3V. The signals from individual phosphopeptides in *A* and a second identical experiment were quantified by densitometry and averages are shown. To eliminate variations in total radioactivity in each sample, the intensity of each phosphopeptide on a given TLC plate was normalized to the intensity of phosphopeptide 2, which did not change in phosphorylation in the control/cAMP conditions.

the phosphopeptide map of NHE3V in control cells (Fig. 1*A*). In contrast, 8-Br-cAMP increased the phosphorylation of two phosphopeptides of NHE3 (Fig. 1*A, 3* and *4, arrowheads*) in cells that contained either NHE-RF or E3KARP (Fig. 1*A*). These results were quantitated in two experiments. The total counts of the IP NHE3 was initially determined by Cerenkov counting, and equal counts were used for the control/cAMP conditions. By analysis of the total counts in the area of the five specific phosphopeptides, the control/cAMP conditions were further normalized with the result that similar total counts were examined for each set of control/cAMP conditions. Visual inspection of the five phosphopeptides revealed that one phosphopeptide, phosphopeptide 2, was most consistent in magnitude of phosphorylation in the control/cAMP conditions for control cells, as well as for NHE-RF- and E3KARP-transfected cells. Consequently, phosphopeptide 2 was used to further nor-

malize the data by comparing the number of counts in each phosphopeptide on a given plate to that in phosphopeptide 2 on the same plate, which was set to 100%. With this normalization, in two experiments phosphopeptides 3 and 4 increased in the presence of cAMP in the NHE-RF- and E3KARP-transfected cells, but in the untransfected cells, these phosphopeptides increased less or not at all. Phosphopeptide 1 did not have consistent changes with cAMP in NHE-RF- and E3KARP-transfected cells, whereas phosphopeptide 5 decreased in control cells, as well as in NHE-RF- and E3KARP-transfected cells. In Fig. 1*B,* we show the changes in each phosphopeptide normalized to phosphopeptide 2 on each plate. Assuming that the changes in phosphorylation of phosphopeptides 3 and 4 caused by cAMP in NHE-RF- and E3KARP-transfected cells were acting by the same mechanisms, we calculated the significance of the cAMP effect on phosphopeptides 3 and 4 using the effects in NHE-RF- and E3KARP-transfected cells as separate experiments. The difference in changes caused by cAMP between NHE-RF/E3KARP-transfected cells compared with in untreated cells for phosphopeptide 3 was 0.14 ± 0.03, $p < 0.025$, and for phosphopeptide 4 was 0.21 ± 0.05, $p < 0.025$.

Confirmatory evidence that cAMP increased NHE3 phosphorylation was provided by *in vitro* back-phosphorylation of NHE3 studied in control conditions and when cells were exposed *in vivo* to 8-Br-cAMP before the *in vitro* phosphorylation with the catalytic subunit of protein kinase A. In PS120/NHE3V control cells not containing NHE-RF, there was no difference in PKA-induced *in vitro* phosphorylation of NHE3V in the presence *versus* in the absence of cAMP *in vivo* ($-1.2 \pm 8.1\%$, $n = 5$, ns) (Fig. 2). In contrast, in cells stably expressing NHE-RF and mutant NHE-RF, PKA caused significantly less phosphorylation of NHE3V *in vitro* in cells exposed *in vivo* to cAMP ($-59.4 \pm 8.9\%$, $n = 5$, $p < 0.005$, and $-65.0 \pm 15.1\%$, $n = 5$, $p < 0.05$, respectively).

cAMP Inhibition of NHE3 Does Not Require NHE-RF Phosphorylation—Further studies evaluated the role of phosphorylation of NHE-RF in the cAMP inhibition of NHE3. Studies were performed only with NHE-RF, because previous studies showed that E3KARP was not phosphorylated under basal conditions or with 8-Br-cAMP exposure (2). PS120/NHE3V cells stably transfected with wild type NHE-RF or NHE-RF/S287AS289A/S290A or not transfected with NHE-RF were metabolically labeled *in vivo* with [^{32}P]orthophosphate and then exposed to 8-Br-cAMP for 15 min. NHE-RF was then immunoprecipitated and separated by SDS-PAGE, and autoradiography and Western analysis were performed on the same samples (Fig. 3). NHE-RF (Fig. 3, *left*) but not the mutant NHE-RF (Fig. 3, *right*) was phosphorylated under basal conditions in cells that were not exposed to 8-Br-cAMP. 8-Br-cAMP exposure did not significantly alter the phosphorylation of NHE-RF (Fig. 3, *left*). There was a $16 \pm 11\%$ increase in NHE-RF phosphorylation in cAMP-treated cells ($n = 3$, p = not significantly different). As shown in Fig. 3, *right,* cAMP did not cause phosphorylation of the mutant NHE-RF.

It was next determined whether mutant NHE-RF was sufficient to allow cAMP to inhibit NHE3 even though mutant NHE-RF was not phosphorylated under basal conditions or in response to cAMP. In Table I, it is shown that 8-Br-cAMP inhibited NHE3 in PS120 cells transfected with mutant NHE-RF. These cells had a rate of intracellular pH recovery of 1.82 ± 0.11 ΔpH_i/min in the absence of cAMP and 1.12 ± 0.03 in the presence of cAMP ($n = 6$, $p < 0.01$). The percentage of inhibition of NHE3 rate caused by cAMP was similar in cells transfected with wild type NHE-RF and mutant NHE-RF (Table I).

NHE3 and NHE-RF Co-Precipitation Is Not Dependent on NHE-RF Phosphorylation or Altered by cAMP Treatment—We

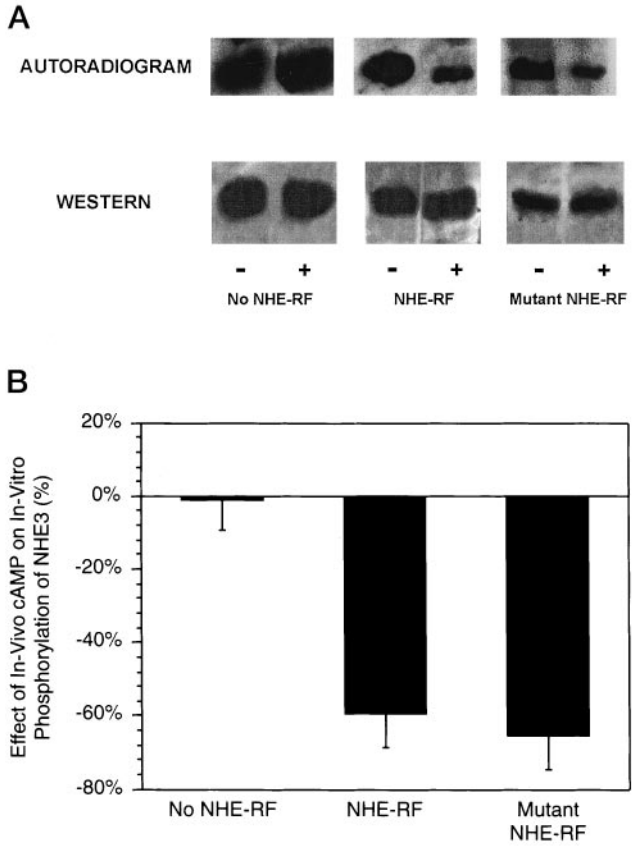

FIG. 2. **8-Br-cAMP alters NHE3 phosphorylation in the presence of NHE-RF and mutant NHE-RF.** *A,* representative autoradiographs (*upper panels*) and Western immunoblots (*lower panels*) of the effect of *in vivo* 8-Br-cAMP exposure on the *in vitro* phosphorylation of immunoprecipitated NHE3. NHE3 was immunoprecipitated from PS120 cells expressing VSVG-tagged NHE3 in the absence (*no NHE-RF*) and in the presence of co-expression of either wild type NHE-RF (*NHE-RF*) or NHE-RF/S287A/S289A/S290A (*mutant NHE-RF*). The cells were studied in the absence of 8-Br-cAMP (−) or following 15 min *in vivo* treatment with 100 μM 8-Br-cAMP (+). The immunoprecipitated NHE3 was then phosphorylated *in vitro* using PKA catalytic subunit and [γ-^{32}P]ATP. A representative experiment is shown; similar results were found in five identical experiments. *B,* results from the five above experiments were analyzed by scanning densitometry/ImageQuant software. The effect of *in vivo* cAMP exposure on *in vitro* PKA-induced phosphorylation was determined as percentage of change in NHE3 phosphorylation. Results shown are mean ± S.E. of cAMP effect (paired *t* test).

previously demonstrated that immunoprecipitation of NHE-RF co-precipitated NHE3. We now show that NHE-RF and NHE3 co-precipitate and that similar co-precipitation occurs in the absence and presence of 8-Br-cAMP (Fig. 4). In addition, when similar experiments were performed with PS120/NHE3V cells stably expressing mutant NHE-RF, mutant NHE-RF and NHE3 co-precipitated. This co-precipitation was similar in control and 8-Br-cAMP-treated cells (Fig. 5).

DISCUSSION

cAMP regulation of the intestinal epithelial brush border Na^+/H^+ exchanger NHE3 occurs physiologically as part of the digestive process, and it occurs in an exaggerated form in diarrheal diseases, such as cholera. Similarly, parathyroid hormone inhibition of renal proximal tubule NHE3 is partially mediated by cAMP (15). Prior studies have shown a requirement in this regulation for members of the regulatory factor gene family NHE-RF or E3KARP. A mechanism suggested as being involved in RF mediation of cAMP inhibition of NHE3 includes action in a signaling complex that includes NHE3,

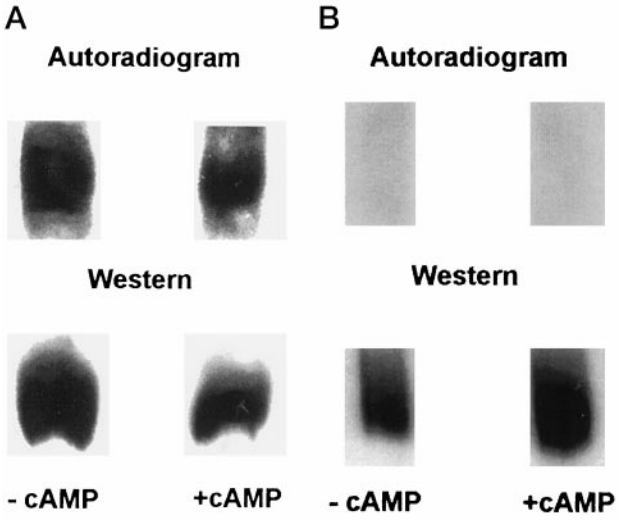

FIG. 3. **8-Br-cAMP does not alter NHE-RF phosphorylation or cause phosphorylation of a mutant NHE-RF.** Representative autoradiograph (*top*) and Western immunoblot (*bottom*) of NHE-RF immunoprecipitated from [^{32}P]orthophosphate-labeled PS120 cells expressing rabbit NHE3V and wild type NHE-RF (*left panel*) or NHE3V and NHE-RF/S287A/S289A/S290A (*right panel*). Studies were performed in the absence (−*cAMP*) and presence (+*cAMP*) of 100 μM 8-Br-cAMP. The bands representing NHE-RF are at 55 kDa. Similar results were found in three identical experiments.

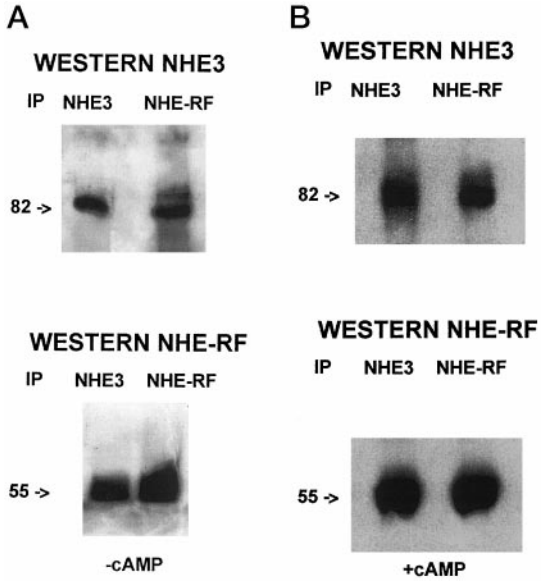

FIG. 4. **NHE-RF co-precipitates NHE3: lack of effect of cAMP.** Representative Western immunoblot demonstrating co-immunoprecipitation of native rabbit NHE-RF and NHE3. PS120 cells were co-transfected with rabbit NHE3V and rabbit NHE-RF. Studies were performed in the absence of cAMP (*left*) or the presence of cAMP (*right*). NHE3 (87 kDa) was resolved using 10% PAGE (*top*), and NHE-RF (55 kDa) was resolved using 6% PAGE (*bottom*). Similar results were found in three identical experiments.

NHE-RF or E3KARP, ezrin, and cAMP-dependent protein kinase II and results in cAMP-dependent phosphorylation of NHE3 (2, 9–12, 16). Previous results indicate that NHE-RF or E3KARP binds to ezrin and to NHE3. Although neither NHE-RF nor E3KARP acts as an A kinase-anchoring protein, ezrin does bind PKA II (2, 15). However, until the current study, it was not established that cAMP-dependent phosphorylation of NHE3 required the presence of NHE-RF or E3 KARP. The current studies were designed to more fully understand the biochemical steps between the activation of PKA and inhibition of NHE3 activity, specifically to determine (i)

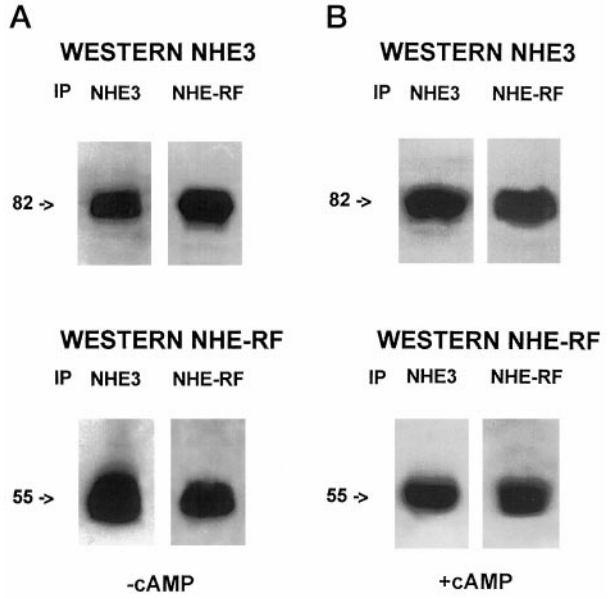

FIG. 5. **Mutant NHE-RF co-precipitates NHE3: lack of cAMP effect.** Representative Western immunoblot demonstrating co-immunoprecipitation of mutant rabbit NHE-RF and NHE3. PS120 cells were co-transfected with rabbit NHE3V and rabbit mutant NHE-RF containing serine to alanine mutations at residues 287, 289, and 290. Studies were performed in the absence (*left*) or presence (*right*) of cAMP. NHE3 was resolved using 10% PAGE (*top*), and NHE-RF was resolved using 6% PAGE (*bottom*). Similar results were found in two identical experiments.

whether NHE-RF or E3KARP was necessary for cAMP to phosphorylate NHE3; and (ii) whether basal or cAMP-stimulated phosphorylation of NHE-RF was important for cAMP inhibition of NHE3 or for NHE-RF to associate with NHE3 as part of a signaling complex.

The present studies confirm our previous observation that the presence of NHE-RF or E3KARP is necessary for cAMP inhibition of NHE3 (1) and show that NHE-RF or E3KARP is necessary for cAMP to phosphorylate NHE3. Using two-dimensional thin layer chromatographic analysis of chymotryptic digests of *in vivo* [^{32}P]orthophosphate-labeled NHE3, these studies show that cAMP did not change the NHE3 phosphorylation pattern in the absence of NHE-RF or E3KARP, whereas a change in two chymotryptic phosphopeptides of NHE3 occurred with cAMP treatment in cells transfected with NHE-RF or E3KARP. In addition, it was shown that cAMP caused changes in the same phosphopeptides in cells containing either NHE-RF or E3KARP. Collectively, these studies demonstrate that in PS120 cells expressing rabbit NHE3, there is an absolute requirement for NHE-RF or E3KARP to demonstrate cAMP inhibition of NHE3, that NHE3 and NHE-RF physically associate *in vivo*, and that NHE-RF or E3KARP is required for PKA-mediated phosphorylation of NHE3.

Others have demonstrated that cAMP causes changes in phosphorylation of NHE3 expressed in fibroblasts or the OK renal proximal tubule cell line (17–19). However, there is some controversy concerning which part of NHE3 is phosphorylated and the significance of the phosphorylation for cAMP inhibition of NHE3. Kurashima *et al.* (17) demonstrated that in AP-1 fibroblasts, cAMP phosphorylates NHE3 on a single serine residue, Ser^{605}. Mutating this serine inhibited cAMP inhibition of NHE3 by 50%. More recently, Zhao *et al.* (19), in studies using the same AP-1 cells as well as OK cells, showed that cAMP phosphorylates NHE3 on multiple serines. In addition, mutating either Ser^{605} or Ser^{552}, greatly decreased cAMP inhibition of NHE3. Mutating three serines other than Ser^{605} and

Ser^{552} also decreased cAMP inhibition of NHE3, an effect potentially due to a larger change in the structure of NHE3 (19). Our results cannot be directly compared with those of Zhao *et al.* (19) or Kurashima *et al.* (17), because different proteases were used. However, our results are most consistent with the studies of Zhao *et al.* (19), because cAMP increased phosphorylation of more than one phosphopeptide in NHE3. Importantly, the data most inconsistent among previous studies of NHE3 phosphorylation relate to the potential role for Ser^{552} in cAMP inhibition of NHE3. Cabado *et al.* (20) used C-terminal truncation mutants of NHE3 to define the domain in the NHE3 cytoplasmic C terminus, which is involved in cAMP inhibition of NHE3 in AP-1 cells. C-terminal truncation to NHE3 amino acid 638 decreased the extent of cAMP inhibition, whereas truncation to amino acid 579 eliminated cAMP regulation of NHE3. On face value, these results are consistent with Ser^{605} but not Ser^{552} being involved in cAMP inhibition because Ser^{552} is N-terminal of the domain of NHE3, which is involved in cAMP regulation of NHE3.

The second major aim of the present experiments was to study the relationship between the phosphorylation of NHE-RF and its function in cAMP inhibition of NHE3. Prior *in vitro* experiments using chemically purified, immunopurified, and recombinant NHE-RF have indicated that NHE-RF was phosphorylated by PKA (2, 4–8). On the other hand, more recent studies using HEK293 and OK cells expressing rabbit NHE-RF have indicated that although NHE-RF is a phosphoprotein in unstimulated cells, treatment of the cells with cAMP resulted in little (HEK293) or no (OK cells) increase in phosphorylation of NHE-RF (2). The relation between the *in vivo* phosphorylation status of NHE-RF and PKA regulation of Na^+/H^+ exchange activity, however, was not determined in those experiments. The present experiments using PS120 cells support the observation that cAMP does not significantly increase the phosphorylation of wild type NHE-RF. These results also indicate that an increase by PKA in NHE-RF phosphorylation is not critical to its function as a co-factor in PKA regulation of NHE3. Although the failure to observe an increase in the phosphorylation of NHE-RF following treatment with cAMP of PS120 cells confirms the findings from studies using HEK293 and OK cells, it remained possible that cAMP phosphorylation of NHE-RF was masked by virtue of the fact that NHE-RF was overexpressed in all three model cell systems. However, this consideration was eliminated by the finding that the S287A/S289A/S290A mutant was not constitutively phosphorylated *in vivo,* nor was its phosphorylation state altered by cAMP, yet the mutant nonphosphorylated form of NHE-RF co-immunoprecipitated NHE3 and was fully active as an accessory co-factor for PKA-mediated inhibition of NHE3. Moreover, mutant NHE-RF facilitated phosphorylation of NHE3, based on back-phosphorylation studies. Collectively, these findings provide strong confirmation for the conclusion that basal or PKA-mediated phosphorylation of NHE-RF is not essential to its function in regulating the relationship between PKA activation and NHE3 activity and NHE3 phosphorylation in PS120 cells or in the physical association between NHE-RF and NHE3.

Several points are worthy of additional discussion. First, the present results indicate that one or more of the NHE-RF serine residues 287, 289, or 290 is phosphorylated in the basal state. At the present time, the functional significance of the basal phosphorylation of NHE-RF is unknown, and the relevant protein kinase has not been identified. Although the phosphorylation of NHE-RF by PKA is not critical to its interaction with NHE3, it remains possible that the phosphorylation of NHE-RF is necessary and required for some of its other physiologic functions, such as those related to its binding to the $\beta 2$ receptor or to CFTR (12, 21). Another issue relates to differences in these *in vivo* findings and previously reported *in vitro* studies of cAMP inhibition of Na^+/H^+ exchange based on reconstitution of renal brush border vesicles. In these *in vitro* studies, PKA phosphorylation of NHE-RF appeared to occur and to be necessary for NHE-RF to act as a co-factor in PKA-mediated inhibition of rabbit renal brush border Na^+/H^+ exchange activity (4–8). In addition, *in vitro* studies using a recombinant protein of NHE-RF/S287A/S289A/S290A and a brush border reconstitution assay indicated that the mutant was not a substrate for PKA but that it also was not functional in mediating an inhibitory effect of PKA on renal brush border Na^+/H^+ exchange (8). At the present time, there is no clear explanation for these differences between the *in vitro* and *in vivo* studies. One potentially relevant difference relates to the study of native rabbit BBM NHE3 from renal epithelial cells *versus* the study of NHE3 transfected into a fibroblast cell line. It may be speculated that if the phosphorylation of NHE-RF by PKA is not critical to its function in allowing PKA phosphorylation of NHE3, basal or cAMP-stimulated NHE-RF phosphorylation might be required to activate or deactivate an additional factor in rabbit solubilized renal brush border membranes; this postulated factor would not be involved in PS120 cells. The more difficult nature of the *in vitro* reconstitution studies must be considered as an additional explanation for these differences.

In summary, the present experiments indicate that NHE-RF or E3KARP is absolutely required for NHE3 inhibition by cAMP when expressed in PS120 cells. NHE-RF and NHE3 are physically associated in these cells, and NHE-RF or E3KARP allows PKA-mediated phosphorylation of NHE3.

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