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Research Article

Inhibition of the renal brush border membrane (BBM) Na/H exchanger by cAMP-dependent protein kinase, PKA, requires participation of a recently cloned regulatory cofactor, Na/H exchanger-regulatory factor (NHE-RF). As deduced from the cDNA of this 358-amino acid protein, amino acids 11-101 and amino acids 150-241 of the NHE-RF protein share 74% overall homology suggesting duplication of these PDZ containing domains. The serine residues at amino acid position 289 and 340 are considered to be the most likely sites for PKA mediated phosphorylation. To study the structure- function relation between NHE-RF and PKA mediated inhibition of the rabbit BBM Na/H exchanger, the effect of recombinant proteins representing full-length NHE-RF as well as truncated and mutant forms of NHE-RF were determined using a reconstitution assay. The reconstitution assay employed a fraction of rabbit BBM proteins that contains Na/H exchanger activity that is not regulated by PKA. NHE-RF in the presence of ATP and Mg but not PKA, inhibited Na/H exchange activity in a concentration-dependent manner. In the presence of PKA, there was a significant left shift in the dose-response relation such that 10(-12) M NHE-RF inhibited Na/H exchange transport by 30% in the presence but not in the absence of PKA. A recombinant polypeptide representing amino acids 1-151 (Domain I) did not affect Na/H exchange transport in the presence or absence of PKA. […]

Structure–function of Recombinant Na/H Exchanger Regulatory Factor (NHE-RF)

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Abstract

Inhibition of the renal brush border membrane (BBM) Na/H exchanger by cAMP-dependent protein kinase, PKA, requires participation of a recently cloned regulatory cofactor, Na/H exchanger–regulatory factor (NHE-RF). As deduced from the cDNA of this 358–amino acid protein, amino acids 11–101 and amino acids 150–241 of the NHE-RF protein share 74% overall homology suggesting duplication of these PDZ containing domains. The serine residues at amino acid position 289 and 340 are considered to be the most likely sites for PKA mediated phosphorylation. To study the structure–function relation between NHE-RF and PKA mediated inhibition of the rabbit BBM Na/H exchanger, the effect of recombinant proteins representing full-length NHE-RF as well as truncated and mutant forms of NHE-RF were determined using a reconstitution assay. The reconstitution assay employed a fraction of rabbit BBM proteins that contains Na/H exchanger activity that is not regulated by PKA. NHE-RF in the presence of ATP and Mg but not PKA, inhibited Na/H exchange activity in a concentration-dependent manner. In the presence of PKA, there was a significant left shift in the dose–response relation such that 10^{-12} M NHE-RF inhibited Na/H exchange transport by 30% in the presence but not in the absence of PKA. A recombinant polypeptide representing amino acids 1–151 (Domain I) did not affect Na/H exchange transport in the presence or absence of PKA. A polypeptide representing amino acids 149–358 (Domain II) in the presence of ATP and Mg but not PKA, inhibited Na/H exchange activity in a concentration-dependent manner. In the presence of PKA, there was a left shift in the dose–response relation. 10^{-12} M of Domain II polypeptide inhibited transport by 18% in the presence but not in the absence of PKA. Mutation of serine residues 287, 289, and 290 to alanine did not affect the inhibitory effect in the absence of PKA but abolished the left shift in the dose–response relation elicited by PKA. Mutation of serine residues 339 and 340 to alanine were without effect on PKA dependent regulation of Na/H exchange transport. These studies indicate that NHE-RF inhibits basal rabbit renal BBM Na/H exchange activity—an effect which is augmented by PKA. The amino acid sequences in the polypeptide containing only the NH_2-terminal PDZ domain of NHE-RF have no intrinsic activity as an inhibitor but appears to be required for the full-length NHE-RF to express its full inhibitory effect on the BBM Na/H exchanger. One or more of the serine residues at positions 287, 289, and/or 290 represent the critical PKA phosphorylation site(s) on the NHE-RF protein that mediates the physiologic effect of cAMP on the renal BBM Na/H exchanger. (*J. Clin. Invest.* 1998. 101:2199–2206.) Key words: renal BBM Na/H exchanger • renal electrolyte transport • Na/H exchanger regulatory cofactor • cAMP-dependent protein kinase

Introduction

It is now well established that activation of cAMP-dependent protein kinase, PKA, inhibits Na/H exchange transport in the renal brush border membrane (BBM)[1] (1–6). In vitro studies using solubilized rabbit renal BBM and a reconstitution assay have suggested that the regulatory effect of PKA can be dissociated from the basal rate of transport (7–10). These observations resulted in the initial isolation of a cofactor necessary for expression of the inhibitory effect of PKA on the renal BBM Na/H exchanger. Subsequently, the cofactor was cloned and designated as the Na/H exchanger–regulatory factor (NHE-RF; reference 11). The cDNA for NHE-RF encodes a protein of 358 amino acids. As predicted from the cDNA, amino acids 11–101 and amino acids 150–241 share 74% overall homology suggesting duplication of this domain. Each domain contains a repeated eight–amino acid sequence as well as a PDZ motif. Potential, albeit nonidealized, consensus sequences for PKA phosphorylation are present toward the COOH-terminal end of the polypeptide. The serine residues at amino acid position 289 and/or 340 were considered to be the most likely phosphorylation sites. While NHE-RF was initially identified as a regulator of renal BBM Na/H exchange activity, unpublished studies suggest that NHE-RF and NHE-RF like proteins may interact with other transporters and hormone receptors (12). Accordingly, there is a need for additional information about the critical structural determinants and interactive domains of the NHE-RF protein that mediates its multiple physiologic functions.

Prior studies have used column chromatography and/or immunoprecipitation to purify NHE-RF—procedures that yielded very limited amounts of protein for detailed study (5, 9, 10). Our experiments were designed to examine the structure–function relation between NHE-RF and PKA-mediated inhibition of the rabbit renal BBM Na/H exchanger using recombinant proteins representing full-length NHE-RF (amino acids 1–358), amino acids 1–151 (Domain 1) containing one of

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1. *Abbreviations used in this paper:* BBM, brush border membrane; HA, hemagglutinin; HEK, human embryonic kidney; NHE-RF, Na/H exchanger–regulatory factor.

the PDZ domains, and amino acids 149–358, a polypeptide that includes the second PDZ domain as well as the COOH-terminal end of NHE-RF (Domain II). These polypeptides were studied using a reconstitution assay containing solubilized renal BBM proteins rendered devoid of NHE-RF activity. Oligonucleotide-directed site-specific mutagenesis was employed to determine the critical phosphorylation site(s) in NHE-RF.

Methods

Recombinant proteins representing NHE-RF (1–356), Domain I (1–151), and Domain II (149–358) of NHE-RF, and NHE-RF with serine to alanine mutations of serine residues 339 and 340 (Mutation I) or serine residues 287, 289, and 290 (Mutation II) were obtained by in-frame ligation of the appropriate cDNA into the pET-30a(+) vector (Novagen, Inc., Madison, WI). The rabbit cDNA for NHE-RF was cut using BSSHII and EcoRI restriction enzymes, the overhangs filled using dNTPs and Klenow reagent, and the inserts blunt ligated into the EcoRV site of the vector. The resulting plasmids were transformed into NovaBlue(DE3) competent cells. Clones were selected for correct orientation by endonuclease restriction mapping. Domain I was made by restricting the pET-NHE-RF plasmid with SacI. To prepare Domain II, the pET-30a vector and the cDNA for NHE-RF were cut with SacI and XhoI. The purified fragments were ligated, transformed into NovaBlue, and verified by restriction mapping. Positive clones were identified for both domains. The plasmids were then isolated, transformed into the expression host BL21, and reconfirmed by restriction mapping.

The mutant forms of NHE-RF were made using a site-specific plasmid DNA mutagenesis kit, MORPH™ (5 Prime 3 Prime, Inc., Boulder, CO) and the mutations confirmed by sequencing. For Mutation I, serine residues 339 and 340 were mutated to alanine residues using an oligonucleotide of the following sequence: 5′-GGCCCG-TCTTGCTGCGCGCTTCTG-3′. For Mutation II, serine residues 287, 289, and 290 were mutated to alanine residues using an oligonucleotide of the following sequence: 5′-GCTGGTGTCAGCGGCG-GCGGCTCTTGC-3′. The positive clones were isolated, verified, and transformed as described above.

All NHE-RF proteins and peptides were expressed as fusion proteins containing a NH_2-terminal hexahistidine tag and were purified by nickel affinity chromatography following the procedure in the pET System Manual (Novagen, Inc.). Where indicated, the fusion proteins were cleaved with recombinant enterokinase (Novagen, Inc.) and the tag was separated from the NHE-RF protein by nickel affinity chromatography.

Western immunoblots were performed by the method of Towbin et al. (13). NHE-3 was detected using a polyclonal antibody to a fusion protein containing the last 85 amino acids of rabbit NHE-3 and glutathione S–transferase (14). NHE-RF proteins and peptides were detected using an affinity-purified antipeptide antibody to a 10–amino acid sequence (KGPNGYGFNL) of NHE-RF that includes an eight–amino acid repeated sequence present in Domain I and Domain II of NHE-RF (10). The proteins were separated initially by SDS-PAGE and electrophoretically transferred to nitrocellulose. The immune complexes were detected by an enhanced chemical luminescence system (Amersham Corp., Arlington Heights, IL).

The methods for preparation of BBM vesicles from the rabbit kidney and the procedures for solubilizing and reconstituting the membrane proteins into artificial liposomes have been described extensively in an earlier publication (7). In brief, BBM vesicles were obtained from the rabbit kidney by a magnesium precipitation method. Membrane proteins were extracted by mixing one part of the membrane preparation (5 mg/ml) with 1.25 parts of 8% octyl glucoside, pH 7.4, for 15 min at 0°C. Frozen and thawed solubilized membrane proteins were applied to a Mono Q HR 5/5 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) anion exchange column and eluted sequentially with 50 mM Tris, 0.1 mM EDTA, 0.1 mM DTT, 2% octyl glucoside, and 0.2 M NaCl and then with the same buffer containing 0.4 M NaCl. The later fraction contains proteins that have Na/H exchange activity that is not regulated by PKA (fraction B; reference 5). The eluted proteins were desalted by Sephadex-G25 chromatography.

Fraction B proteins were mixed with individual recombinant proteins in the presence of ATP (50 μM) and Mg (200 μM) for two minutes at 30°C before reconstitution. Controls for these experiments were the recombinant proteins denatured in a boiling water bath for 10 min followed by rapid cooling on ice. The concentrations of the denatured proteins were the same as those of the native proteins used in the corresponding experiments. Where indicated, the catalytic subunit of PKA (50 U) was added to this phosphorylation solution. The membrane-detergent mixture was centrifuged at 100,000 *g* for 30 min. 1.6 parts of the supernatant were mixed with one part of asolectin (35 mg/ml; crude soybean phospholipid; Associated Concentrates, Woodside, NY) which had been sonicated to translucency for 10 min at 22°C. Proteoliposomes were prepared by dialysis for 18 h at 4°C using 12,000–14,000 D cutoff membranes. The dialysis buffer contained 250 mM mannitol, 50 mM MES/Tris, and 30 mM potassium gluconate, pH 6.0. The Na/H exchange rate was determined in the reconstituted proteoliposomes by measurement of proton gradient simulated uptake of sodium (7). The internal pH of 6.0 was set by the dialysis solution. The final uptake solution contained 1 mM ^{22}Na, 250 mM mannitol, 30 mM potassium gluconate, 1 g/ml valinomycin, and 50 mM Tris/MES to set pH to 8.0 or 6.0. Sodium uptake was determined by applying the reaction mixture to 1 ml Dowex 50 × 8 (Tris), 100-mesh columns and rapid elution with vacuum suction with 1 ml of 300 mM sucrose, pH 8.0, at 0°C. Under these voltage clamped conditions (K_{in} = K_{out} = 30 mM, valinomycin), the proton stimulated component of sodium uptake was taken as a measure of the Na/H exchange rate.

To determine the in vivo phosphorylation of NHE-RF, human embryonic kidney (HEK) 293 cells, obtained from American Type Culture Collection (ATCC, Rockville, MD), were transfected with 2 μg of pBK-CMV (Stratagene, Inc., La Jolla, CA) containing the NHE-RF cDNA using the calcium-phosphate method. This expression plasmid of hemagglutanin (HA)-tagged NHE-RF was constructed as described previously (15). The cells were maintained in 75% DME supplemented with 10% (vol/vol) heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml; GIBCO BRL, Gaithersburg, MD) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. 48 h after transfection, the medium was replaced with 6 ml of phosphate-free DME supplemented 0.5% (vol/vol) heat-inactivated FCS and 0.4 mCi [^{32}P]orthophosphate. Where studied, the cells were treated with 0.1 mM 8-bromo-cylic adenosine monophosphate (0.1 mM) for 10 min. After 90 min, the cells were washed three times with 6 ml of Dulbecco's PBS and then scrapped into 1 ml of ice cold lysis buffer containing 0.1% SDS, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 50 nM calyculin, 1 mM benzamide, and 100 μM phenylmethanesulfonyl fluoride. The lysates were transferred to 1.5 ml microcentrifuge tubes, rocked for 30 min at 4°C, and centrifuged at 100,000 *g* for 4 min. 1 ml of supernatant was transferred to a 1.5-ml microcentrifuge tube and 6 μg of 12CA5 monoclonal antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) added. After rocking at 4°C for 60 min, 60 μl of protein A plus protein G agarose (Calbiochem Corp., La Jolla, CA) was added and the samples rocked for an additional 60 min at 4°C. The protein A–Sepharose was then washed three times in lysis buffer after which 100 μl of SDS-sample buffer was added and the samples heated to 85°C for 10 min. The proteins were resolved on 12% polyacrylamide slab gels and the phosphoproteins visualized by autoradiography.

Protein concentrations were determined by the method of Lowry et al. (16). Results of experiments are presented as the mean of means±SEM. Statistical comparisons between control and experimental samples was performed using Peritz Analysis of Variance (17). Representative examples of SDS-PAGE slab gels, autoradiographs, and Western hybridization analyses are shown.

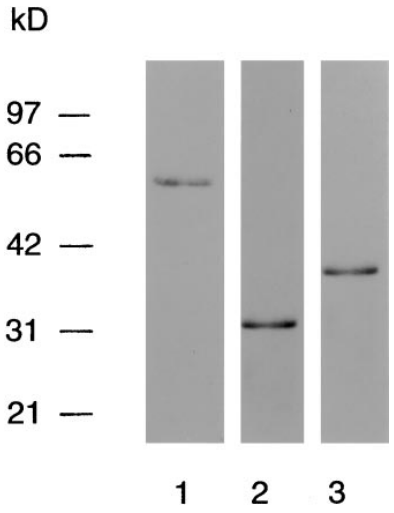

Figure 1. SDS-PAGE gel of recombinant proteins representing NHE-RF (lane *1*), Domain I (lane *2*), and Domain II (lane *3*).

Results

Fig. 1 is an SDS-PAGE of recombinant full-length NHE-RF (lane *1*), recombinant polypeptides representing Domain I (lane *2*), and Domain II (lane *3*) purified by nickel affinity chromatography. The activity of the recombinant proteins was measured using a reconstitution assay. The recombinant proteins were added to an anionic fraction of frozen and thawed solubilized rabbit BBM proteins (fraction B). This membrane fraction previously has been shown to have Na/H exchange activity that is not regulated by PKA and one that lacks NHE-RF (5). Preliminary experiments established that heat inactivated recombinant proteins in the presence or absence of PKA, had no effect on Na/H exchange activity. All the recombinant proteins contained a 52–56 amino acid leader sequence having a His Tag and an enterokinase site. This leader sequence contained also a putative PKA phosphorylation site. Two lines of evidence indicated that the presence of the tag did not affect the activity of the recombinant proteins. First, the recombinant protein representing Domain I contained the tag but did not affect Na/H exchange activity (see Table II). Second, the activity of recombinant NHE-RF containing the tag was compared with recombinant NHE-RF in which the tag was cleaved using enterokinase. The activity of NHE-RF lacking the tag was identical to that of the NHE-RF fusion protein. In the absence of PKA, Na/H exchange activity was 26.3±3.6 (pmol/mg protein/2 min) using the cleaved NHE-RF protein (10^{-12} M) and 24.2±3.3 using the fusion protein (10^{-12} M; n = 4). PKA inhibited Na/H exchange activity by 29.7±4.0% using the cleaved protein and 28.0±0.8% using the fusion protein. In view of these results and the potential for degradation of recombinant NHE-RF due to the extensive enterokinase digestion required for complete cleavage, all remaining studies were performed using recombinant tagged proteins. The relative abundance of NHE-3 and recombinant NHE-RF including the truncated and mutated forms of NHE-RF incorporated into the proteoliposomes was determined by Western immunoblot analysis of proteoliposomes after reconstitution of the solubilized BBM proteins in the presence of ATP and Mg. Fig. 2 *A* demonstrates the abundance of rabbit NHE-3 did not differ

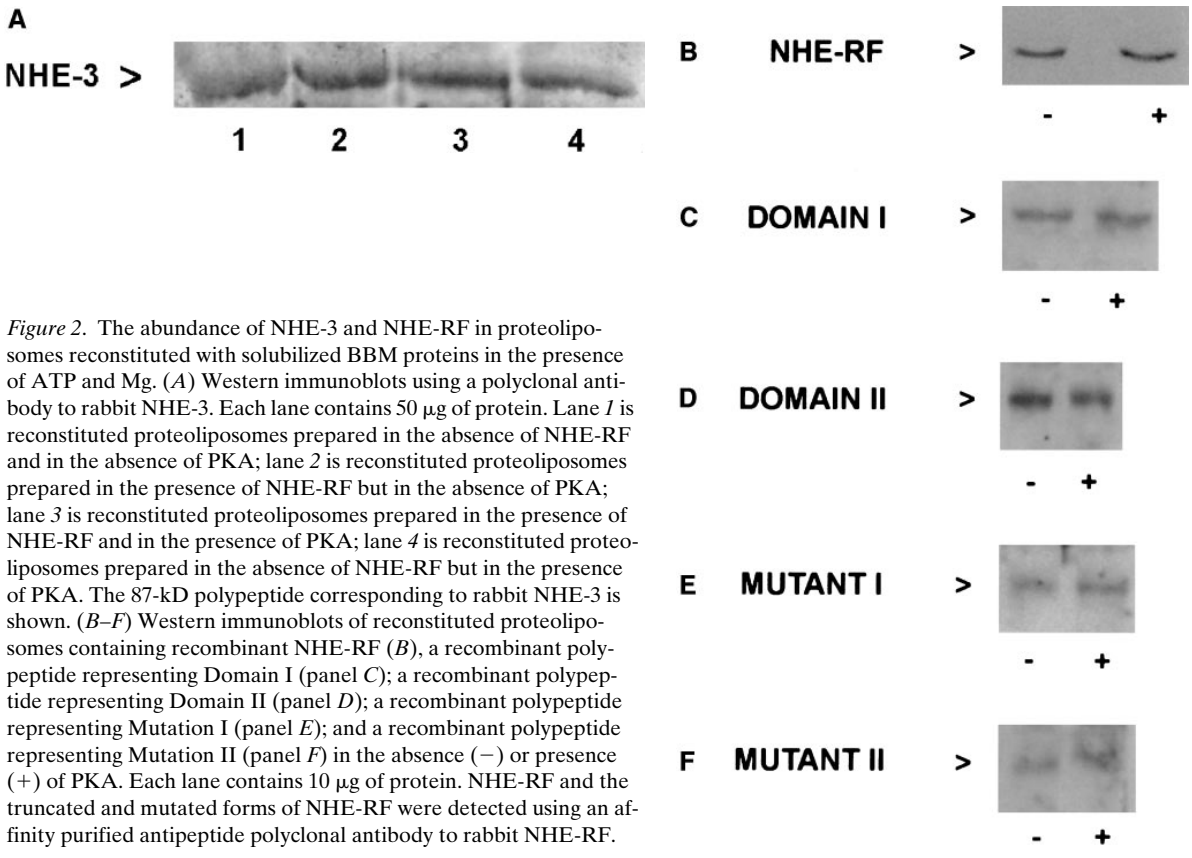

Figure 2. The abundance of NHE-3 and NHE-RF in proteoliposomes reconstituted with solubilized BBM proteins in the presence of ATP and Mg. (*A*) Western immunoblots using a polyclonal antibody to rabbit NHE-3. Each lane contains 50 μg of protein. Lane *1* is reconstituted proteoliposomes prepared in the absence of NHE-RF and in the absence of PKA; lane *2* is reconstituted proteoliposomes prepared in the presence of NHE-RF but in the absence of PKA; lane *3* is reconstituted proteoliposomes prepared in the presence of NHE-RF and in the presence of PKA; lane *4* is reconstituted proteoliposomes prepared in the absence of NHE-RF but in the presence of PKA. The 87-kD polypeptide corresponding to rabbit NHE-3 is shown. (*B–F*) Western immunoblots of reconstituted proteoliposomes containing recombinant NHE-RF (*B*), a recombinant polypeptide representing Domain I (panel *C*); a recombinant polypeptide representing Domain II (panel *D*); a recombinant polypeptide representing Mutation I (panel *E*); and a recombinant polypeptide representing Mutation II (panel *F*) in the absence (−) or presence (+) of PKA. Each lane contains 10 μg of protein. NHE-RF and the truncated and mutated forms of NHE-RF were detected using an affinity purified antipeptide polyclonal antibody to rabbit NHE-RF.

significantly when proteoliposomes were prepared in the absence of NHE-RF and PKA (lane *1*), in the presence of NHE-RF but absence of PKA (lane *2*), in the presence of both NHE-RF and PKA (lane *3*), and in the presence of PKA alone (lane *4*). As shown in Fig. 2, *B–F*, the relative abundance of recombinant full-length NHE-RF (Fig. 2 *B*), Domain I (Fig. 2 *C*), Domain II (Fig. 2 *D*), Mutation I (Fig. 2 *E*), or Mutation II (Fig. 2 *F*) was not different in the absence (−) or presence (+) of PKA.

As compared with heat-inactivated NHE-RF used in the same concentration, recombinant NHE-RF in the presence of ATP and Mg but not PKA, inhibited Na/H exchange activity in a concentration-dependent manner (Fig. 3). Additional preliminary studies established that the baseline inhibitory effect of NHE-RF did not require the presence of ATP (data not shown). In the presence of PKA, there was a significant left shift in the dose–response relation. As summarized in Table I, NHE-RF in a concentration of 10^{-12} M did not inhibit Na/H exchange activity in the absence of PKA. In the presence of PKA, however, there was a 30% decrease in the proton gradient–stimulated component of Na uptake, a measure of Na/H exchange activity. The truncated polypeptide representing the NH_2-terminal region of NHE-RF, Domain I, had no effect on Na/H exchange activity in the presence or absence of PKA (Fig. 4 and Table II). The polypeptide representing the COOH-terminal region of NHE-RF, Domain II, inhibited Na/H exchange activity in a concentration dependant manner in the absence of PKA (Fig. 4). PKA mediated phosphorylation was associated with a left shift in the dose–response relation. Domain II in a concentration of 10^{-12} M did not inhibit Na/H exchange activity in the absence of PKA but was associated with a 18% decrease in activity in the presence of PKA (Table III).

To determine the critical functional phosphorylation sites in NHE-RF, serine residues 339 and 340 (Mutation I) or serine residues 287, 289, and 290 (Mutation II) were mutated to alanine residues. Fig. 5 is a representative autoradiogram of recombinant NHE-RF and mutant fusion proteins cleaved with enterokinase and phosphorylated in vitro using Y-[^{32}P]ATP and PKA. NHE-RF and Mutation I were substrates for PKA. Mutation II, however, was not phosphorylated by PKA. These results indicate that PKA-mediated phosphorylation of NHE-RF occurs at serine residues 287, 289, and/or 290. In the absence of PKA, both mutated polypeptides inhibited Na/H exchange activity in a concentration dependent manner (Fig. 6). However, in the presence of PKA, Mutation I (Fig. 6 and Table IV) but not Mutation II (Fig. 6 and Table V) demonstrated a left shift in the dose–response relation between the concentration of the polypeptide and PKA mediated inhibition of Na/H exchange transport.

Metabolic labeling of HEK 293 cells transfected with an epitope-tagged NHE-RF was used to study the phosphorylation of this protein in intact cells. As shown in the representative autoradiograph (Fig. 7), the CA5 monoclonal antibody directed against the hemagglutinin peptide tag immunoprecipitated a 52-kD phosphoprotein representing NHE-RF only

Table I. Effect of Recombinant NHE-RF (10^{-12} M) on Na Uptake into Reconstituted Proteoliposomes (pmol/mg protein/ 2 min)

	Inactive NHE-RF no PKA	Inactive NHE-RF PKA	Active NHE-RF no PKA	Active NHE-RF PKA
8/6	56.2±2.2	55.8±1.6	55.6±6.5	45.5±3.8
6/6	29.8±2.3	28.9±2.8	28.7±3.7	27.7±2.1
Delta	26.5±4.9	26.9±2.6	25.9±2.4	17.8±1.9*

Summary of the uptake of 1 mM Na into reconstituted proteoliposomes containing fraction B proteins from rabbit renal BBM and a polypeptide representing recombinant NHE-RF(10^{-12} M) under chemically voltage clamped conditions in the presence (*8/6*) and absence (*6/6*) of a pH gradient. Delta is the difference between these values and is taken as a measure of the Na/H exchange rate. Results are expressed as the mean of means±SEM. *$P \leq 0.05$.

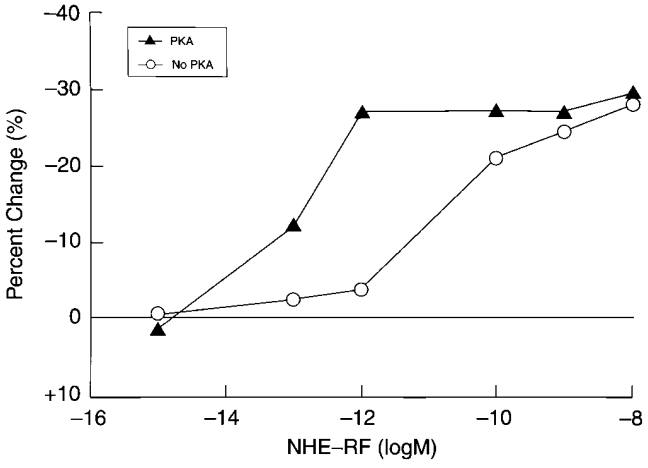

Figure 3. The relation between the amount of recombinant NHE-RF and Na/H exchange activity assayed in reconstituted proteoliposomes in the presence of ATP and Mg, and in the presence of absence of PKA. Results are expressed as percent change from the corresponding heat inactivated recombinant protein (controls).

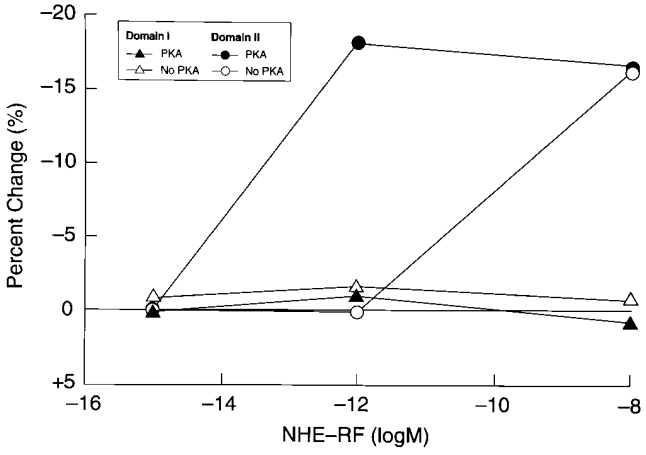

Figure 4. The relation between the amount of recombinant polypeptides representing either Domain I or Domain II of NHE-RF and Na/H exchange activity assayed in reconstituted proteoliposomes in the presence of ATP and Mg, and in the presence or absence of PKA. Results are expressed as percent change from the corresponding heat inactivated recombinant proteins (controls).

Table II. Effect of a Polypeptide Representing Domain I of NHE-RF (10^{-12}M) on Na Uptake into Reconstituted Proteoliposomes (pmol/mg protein/2 min)

	Inactive Domain I no PKA	Active Domain I no PKA	Active Domain I PKA
8/6	56.2±2.2	57.9±1.6	57.9±3.2
6/6	30.6±3.3	33.2±1.2	33.6±1.9
Delta	25.5±1.3	24.7±1.6	24.3±1.5

Summary of the uptake of 1 mM Na into proteoliposomes coreconstituted with fraction B proteins from rabbit renal BBM and a polypeptide representing amino acids 1–151 (Domain I) of NHE-RF (10^{-12} M). Uptake was determined into chemically voltage clamped proteoliposomes in the presence (*8/6*) and absence (*6/6*) of a pH gradient. Delta is the difference between these values and is taken as a measure of the Na/H exchange rate. Results are expressed as the mean of means±SEM.

from transfected cells (lanes *2* and *3*). No radioactive bands were recovered from nontransfected cells (lane *1*). As compared with transfected cells not treated with cAMP (lane *2*), treatment of the cells with 0.1 mM 8-bromo-cAMP cAMP increased the phosphorylation of NHE-RF by 30% (lane *3*). The equal loading of the lanes was confirmed using a rabbit polyclonal antibody to full-length recombinant NHE-RF. Two other experiments yielded similar results.

Discussion

Studies using solubilized rabbit renal BBM proteins and a reconstitution assay have indicated that the inhibitory effect of PKA on Na/H exchange activity requires the presence of a cofactor distinct from the transporter itself (5, 8, 9, 10). This conclusion derived from the results of studies in which basal Na/H exchange transport activity was dissociated from its regulation by PKA under several different experimental circumstances. Coreconstitution studies resulted in the initial isolation of this cofactor (5, 9). Subsequently, the cofactor was cloned from a rabbit renal cDNA library and named NHE-RF (11). Experiments using PS 120 cells stably expressing rabbit NHE-3 indicated that cAMP did not modulate Na/H exchange activity. By

Table III. Effect of a Polypeptide Representing Domain II of NHE-RF (10^{-12} M) on Na Uptake into Reconstituted Proteoliposomes (pmol/mg protein/2 min)

	Inactive Domain II no PKA	Active Domain II no PKA	Active Domain II PKA
8/6	59.9±4.9	59.7±8.1	54.8±6.6
6/6	34.1±4.5	34.8±5.1	34.4±5.2
Delta	25.8±0.4	26.8±2.4	20.5±1.1*

Summary of the uptake of 1 mM Na into proteoliposomes coreconstituted with fraction B proteins from rabbit renal BBM and a polypeptide representing amino acids 149–358 (Domain II) of NHE-RF (10^{-12} M). Uptake was determined into chemically voltage clamped proteoliposomes in the presence (*8/6*) and absence (*6/6*) of a pH gradient. Delta is the difference between these values and is taken as a measure of the Na/H exchange rate. Results are expressed as the mean of means±SEM. *$P \leq 0.05$.

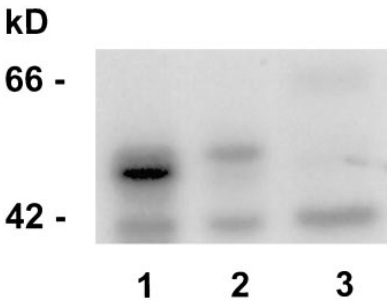

Figure 5. Autoradiogram of a SDS-PAGE of NHE-RF (lane *1*), Mutation I (lane *2*), and Mutation II (lane *3*). The tag of the fusion proteins were cleaved with enterokinase, after which the proteins were phosphorylated in vitro using Y-[^{32}P]STP and PKA. Molecular mass markers (*kD*) are shown. The band at ~ 40 kD represents autophosphorylated PKA.

contrast, cAMP inhibited Na/H exchange activity in PS 120 cells cotransfected with NHE-3 and rabbit NHE-RF (18). Based on the in vitro and in vivo experiments as well as preliminary experiments that suggest that NHE-RF and NHE-RF like proteins regulate Na/H exchange transport as well as other cell functions, these studies were designed to establish the critical interactive domains of NHE-RF as a regulator of PKA mediated inhibition of rabbit renal BBM Na/H exchange transport activity (12).

NHE-RF has a unique structure with significant internal homology between amino acids 11–101 and amino acids 150–241 including an identical eight–amino acid sequence in the two domains. Each domain contains a signature PDZ motif. Recombinant NHE-RF, truncated forms of NHE-RF representing the NH_2-terminal portion of NHE-RF including amino acids 1–151 (Domain I), the COOH-terminal end of the protein encompassing amino acids 149–358 and including potential PKA phosphorylation sites (Domain II), and mutant forms that lacked some of the candidate serine residues of NHE-RF were prepared using the pET vector expressed in *Escherichia coli*. The activity of the purified recombinant proteins were as-

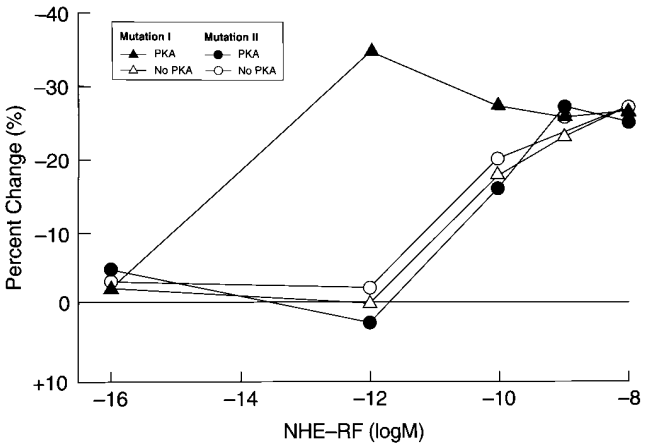

Figure 6. The relation between the amount of recombinant mutated polypeptides representing serine to alanine mutations at either residues 339 and 340 (Mutation I), or residues 287, 289, and 290 (Mutation II) of NHE-RF and Na/H exchange activity assayed in reconstituted proteoliposomes in the presence of ATP and Mg, and in the presence or absence of PKA. Results are expressed as percent change from the corresponding heat inactivated recombinant proteins (controls).

Table IV. Effect of a Polypeptide Representing NHE-RF Containing Serine to Alanine Mutations at Residues 339 and 340 (Mutation I; 10^{-12} M) on Na Uptake into Reconstituted Proteoliposomes (pmol/mg protein/2 min)

	Inactive Mutation I no PKA	Active Mutation I no PKA	Active Mutation I PKA
8/6	53.7±6.1	51.1±5.2	38.8±3.3
6/6	27.2±4.4	24.3±3.2	21.0±2.6
Delta	26.4±3.4	26.8±3.1	17.8±2.3*

Summary of the uptake of 1 mM Na into proteoliposomes coreconstituted with fraction B proteins from rabbit renal BBM and a polypeptide representing NHE-RF containing serine to alanine mutations at residues 339 and 340 (Mutation I; 10^{-12} M). Uptake was determined into chemically voltage clamped proteoliposomes in the presence (*8/6*) and absence (*6/6*) of a pH gradient. Delta is the difference between these values and is taken as a measure of the Na/H exchange rate. Results are expressed as the mean of means±SEM. $*P \leq 0.05$.

sayed using solubilized rabbit renal BBM proteins that had been first freeze-thawed and then separated by anion exchange chromatography. The more anionic fraction of these proteins (fraction B) was coreconstituted with the recombinant NHE-RF polypeptides. This preparation of solubilized rabbit renal BBM proteins as an assay system for study of the activity of the recombinant proteins confers several experimental advantages. First, solubilized rabbit renal BBM proteins have no measurable intrinsic protein kinase or protein phosphatase activity. The lack of intrinsic protein kinase or phosphatase activity facilitates analysis of the functional effect of specific protein kinases such as that mediated by PKA as in the present experiments (8). Second, freezing and thawing of solubilized BBM proteins dissociates Na/H exchange activity from its regulation by PKA allowing convenient analysis of potential regulators (8). Finally, fraction B does not contain endogenous NHE-RF which elutes at lower concentration of salt from the anion exchange column enhancing the analysis of the recombinant NHE-RF polypeptides (5).

Table V. Effect of a Polypeptide Representing NHE-RF Containing Serine to Alanine Mutations at Residues 287, 289, and 290 (Mutation II; 10^{-12} M) on Na Uptake into Reconstituted Proteoliposomes (pmol/mg protein/2 min)

	Inactive Mutation II no PKA	Active Mutation II no PKA	Active Mutation II PKA
8/6	51.7±5.1	50.7±8.1	52.0±4.6
6/6	25.0±2.5	21.7±3.1	21.0±2.2
Delta	26.7±2.1	29.0±2.4	31.1±2.8

Summary of the uptake of 1 mM Na into proteoliposomes coreconstituted with fraction B proteins from rabbit renal BBM and a polypeptide representing NHE-RF containing serine to alanine mutations at residues 287, 289, and 290 (Mutation II; 10^{-12} M). Uptake was determined into chemically voltage clamped proteoliposomes in the presence (*8/6*) and absence (*6/6*) of a pH gradient. Delta is the difference between these values and is taken as a measure of the Na/H exchange rate. Results are expressed as the mean of means±SEM.

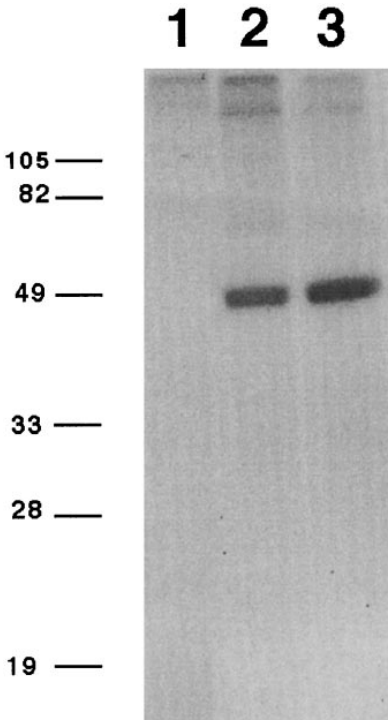

Figure 7. Representative autoradiograph of a SDS-PAGE slab gel of immunoprecipitated HA-tagged NHE-RF from [^{32}P]-orthophosphate metabolically labeled HEK 293 cells expressing HA-tagged NHE-RF in the absence (lane *2*) or presence (lane *3*) of treatment with 0.1 mM 8-bromo-cAMP for 10 min. Lane *1* represents cells not transfected with HA-tagged NHE-RF.

Heat inactivated NHE-RF had no effect on Na/H exchange activity either alone or in the presence of PKA. By contrast, NHE-RF in the presence of ATP and Mg, but without PKA, inhibited Na/H exchange activity in a concentration-dependent manner. Maximal inhibition of $\sim$ 30% was observed at concentrations of NHE-RF of 10^{-10} M or greater. At these concentrations, the addition of PKA did not affect further the rates of Na uptake. In the presence of ATP, Mg, and PKA, however, there was a marked left shift in the dose–response relation such that maximal inhibition of transport activity of $\sim$ 30% was observed using a concentration of NHE-RF of 10^{-12} M. At this concentration, NHE-RF had no effect on Na/H exchange transport in the absence of PKA. The effect of NHE-RF was exclusively on the pH gradient–stimulated component of Na uptake indicating its interaction with the Na/H exchanger. The inhibitory value of 30% is similar in magnitude to prior studies using partially purified rabbit kidney NHE-RF or immunopurified NHE-RF suggesting that this represents the maximal response to PKA under the conditions of study and was not limited by the availability of NHE-RF protein (8). In addition, the above results confirm some of the predictions made in prior studies. Earlier experiments indicated that limited proteolysis mediated by trypsin stimulated basal Na/H exchange activity and dissociated the activity of the transporter from its regulation by PKA (9). These experiments suggested that NHE-RF was an inhibitory polypeptide and that its inactivation by trypsin resulted in increased transporter activity. It was speculated further that PKA mediated phosphorylation of NHE-RF enhanced its inhibitory effect on Na/H exchange activity (9). These experiments are consistent with these speculations.

The recombinant polypeptide representing Domain I, the more proximal NH_2-terminal end of NHE-RF, had no effect on the Na/H exchanger in the presence or absence of PKA in

concentrations up to 10^{-8} M. By contrast, the recombinant polypeptide representing Domain II exhibited the same pattern of response as the full-length NHE-RF. Domain II in a concentration of 10^{-12} M inhibited Na/H exchange activity by $\sim$ 20% in the presence of PKA. Although experiments were not paired, the effect of the Domain II recombinant polypeptide appeared to be qualitatively the same as full-length NHE-RF but differed in magnitude; Domain II was associated with maximal inhibition of transport of 20% as compared with 30% with full-length NHE-RF. These data suggest that the amino acid sequences in Domain I by themselves, do not affect PKA mediated inhibition of the BBM Na/H exchange process, but are, nevertheless, required for the full expression of the inhibitory effect of NHE-RF.

As determined using in vitro techniques, it has been demonstrated that NHE-RF is a substrate for PKA (10). NHE-RF contains two potential, albeit, nonidealized PKA phosphorylation sites. Site direct mutagenesis studies established that the serine residues at positions 287, 289, and/or 290 are critical for in vitro phosphorylation by PKA. Functional studies using the reconstitution assay established that these residues were critical for PKA mediated inhibition of Na/H exchange activity. These residues, however, were not required for expression of the basal inhibitory activity of the NHE-RF protein. Taken together, these data demonstrate that serine residues 287, 289, and/or 290 represent the phosphorylation site(s) necessary for the biologic effect of PKA on the renal BBM Na/H exchanger.

Studies from our laboratory using either PS 120 cells expressing rabbit NHE-3, native rabbit renal BBM, or solubilized BBM proteins and a reconstitution assay have established that the inhibitory effect of PKA on Na/H exchange transport involves a reversible phosphorylation reaction as well as the presence NHE-RF (4, 8, 18). Currently, however, the precise relation between the phosphorylation of NHE-RF and NHE-3, and the inhibition of renal BBM Na/H exchange transport by PKA in vivo has not been established. Studies by Moe et al. (19) have indicated that the phosphorylation of rat NHE-3 expressed in AP-1 cells is increased by activation of PKA. Recent studies by Kurashima et al. (20) established that serine residue 605 of rat NHE-3 is the critical PKA phosphorylation site associated with PKA mediated inhibition of the transporter expressed in AP-1 cells. The seeming disparity between the effect of PKA on rat NHE-3 activity expressed in AP-1 cells and rabbit NHE-3 expressed in PS 120 cells may relate to the fact that CHO cells, the parent cell line of AP-1 cells, are rich in a protein readily identified by an antibody to rabbit NHE-RF while PS 120 cells are devoid of such a protein (18).

Our prior studies, as well as these studies, establish that NHE-RF is an in vitro substrate for PKA. Moreover, NHE-RF is required for PKA-mediated inhibition of NHE-3 activity in PS 120 cells (18). Accordingly, our working hypothesis has been that NHE-RF is phosphorylated by PKA in vivo and that this phosphorylation is critical to expression of the physiologic effect of PKA on the BBM Na/H exchanger. While the effect of PKA on the in vivo phosphorylation of NHE-3 has been established, the in vivo phosphorylation of NHE-RF has not been determined. To begin to address this question, HEK 293 cells were transfected with hemagglutinin-tagged rabbit NHE-RF, which was readily identified using the CA5 monoclonal antibody. Immunoprecipitation experiments indicated that NHE-RF was a phosphoprotein even under conditions where exogenous cAMP was not added. Further treatment of the cells with a cAMP analog resulted in a 30% increase in NHE-RF phosphorylation. Similar data have been obtained with endogenous NHE-RF immunoprecipitated from metabolically labeled OK cells using a polyclonal antibody to full-length recombinant NHE-RF. These and other preliminary studies suggest that NHE-RF is subject to phosphorylation by multiple and as yet unidentified protein kinases in the intact cell. The effect of these phosphorylations on NHE-RF function and/or its subsequent phosphorylation by PKA remains unknown. These results raise the intriguing possibility that NHE-RF is extensively phosphorylated and activated in unstimulated cells, and that the primary target(s) of the inhibitory effects of PKA on Na/H exchange activity in intact cells may include phosphoproteins other than NHE-RF. It is recognized that other alternate hypotheses have not been excluded at this time. For example, it is possible that there are distinct pools of NHE-RF that are differentially phosphorylated by PKA or that the interaction between NHE-RF and Na/H exchange activity includes as yet other unidentified phosphoproteins. Currently, however, we favor the simpler possibility that cellular NHE-RF is phosphorylated by multiple protein kinases and that the basal phosphorylation contributes the relatively modest increase seen after cAMP stimulation. It is clear, however, that further studies will be required to identify the in vivo phosphorylation sites in NHE-RF and to determine the role of specific proteins and protein kinases involved in PKA regulation the renal BBM Na/H exchanger. Taken together with other data, it appears that both NHE-3 and NHE-RF are phosphoproteins in the intact cell. This suggests a complex mechanism for PKA regulation that involves the phosphorylation of the two proteins and their physical interaction to regulate of Na/H exchange transport.

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