Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 3010–3015, April 1997
Cell Biology

cAMP-mediated inhibition of the epithelial brush border Na^+/H^+ exchanger, NHE3, requires an associated regulatory protein

(sodium–hydrogen antiporter/pH regulation/signal transduction/Na^+ absorption)

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Communicated by Gerhard Giebisch, Yale University School of Medicine, New Haven, CT, January 17, 1997 (received for review October 17, 1996)

ABSTRACT **NHE3 is the Na^+/H^+ exchanger located on the intestinal and renal brush border membrane, where it functions in transepithelial Na^+ absorption. The brush border Na^+ absorptive process is acutely inhibited by activation of cAMP-dependent protein kinase, but the molecular mechanism of this inhibitory effect is poorly understood. We have identified two regulatory proteins, E3KARP and NHERF, that interact with NHE3 to enable cAMP to inhibit NHE3. The two regulatory proteins are structurally related, sharing ≈50% identity in amino acid sequences. It has been previously shown that when NHE3 is transfected into PS120 fibroblasts or Caco-2 cells, cAMP failed to inhibit NHE3 activity. Northern blot analysis showed that both PS120 and Caco-2 cells lacked the expression of both E3KARP and NHERF. In contrast, other cell lines in which cAMP inhibits NHE3, including OK, CHO, and LLC-PK_1 cells, expressed NHERF-related regulatory proteins. To determine their functions in cAMP-dependent inhibition of NHE3, E3KARP and NHERF were transfected into PS120/NHE3 fibroblasts. Transfection in PS120/NHE3 fibroblasts with either NHERF or E3KARP reconstituted cAMP-induced inhibition of NHE3, resulting in 25–30% inhibition in these cells.**

Increased cAMP elicited by enterotoxins and some neurotransmitters results in stimulation of net Cl^- secretion and inhibition of electroneutral NaCl absorption in intestinal epithelium (1). Cl^- secretion and inhibition of NaCl absorption by the apical Na^+/H^+ exchanger primarily occur in the crypt and villus epithelial cells, respectively (1). Although inhibition of NaCl absorption and the apical Na^+/H^+ exchanger in the small intestine is a major contributor to diarrhea, very little is known about the molecular mechanisms of the cAMP-dependent inhibition of the absorptive process. Similarly, increased cAMP levels in renal proximal convoluted tubules are associated with inhibition of the apical membrane Na^+/H^+ exchanger (2).

NHE3 is the small intestinal as well as renal proximal tubule brush border Na^+/H^+ exchanger that is involved in transepithelial NaCl and $NaHCO_3$ absorption (see ref. 3 for review). All Na^+/H^+ exchangers respond to a variety of stimuli, including intracellular alkalinization, growth factors, hormones, tumor promoters, and hypertonic stress, and the different biological effects resulting from such stimuli are believed to be mediated by activation of protein kinases (1, 4). It was initially speculated that the regulation of Na^+/H^+ exchangers by these stimuli is mediated by phosphorylation of the exchanger. This speculation is based on the facts that most of the biological stimuli affecting Na^+/H^+ exchangers are believed to be mediated by protein kinases, and the regulation of Na^+/H^+ exchangers was shown to be dependent on the availability of cellular ATP (5–8).

However, recent studies showed that the protein kinase-induced regulation does not always correlate with changes in phosphorylation levels of the Na^+/H^+ exchanger and that phosphorylation of the Na^+/H^+ exchanger alone cannot account for its regulation (6, 9). Wakabayashi *et al.* (9) showed that deletion of all major phosphorylation sites in the cytoplasmic tail of NHE1 failed to completely obliterate the protein kinase stimulation. In addition, we have recently observed that the stimulation of NHE3 by fetal bovine serum and fibroblast growth factor and inhibition by phorbol ester in PS120 cells did not affect the phosphorylation level of NHE3 (J. Yip, M.D., C.T., unpublished data). These observation led to a hypothesis that, at least in some cases, Na^+/H^+ exchangers are regulated via accessory regulatory proteins that mediate the cellular signals between protein kinases and the exchangers.

Therefore, we initiated a study to identify some of the proteins interacting with NHE3 by using a yeast two-hybrid system. One of the clones obtained, E3KARP, showed a high homology with the protein, NHERF, previously identified by Weinman *et al.* (11, 12). NHERF was identified by cellular fractionation of rabbit renal brush border vesicles, and has been shown to reconstitute cAMP-dependent inhibition of the brush border Na^+/H^+ exchanger in renal brush border vesicles *in vitro* (11). We show that both E3KARP and NHERF can reconstitute the cAMP-induced inhibition of NHE3 in a cell culture system, demonstrating the requirement for the presence of a regulatory protein.

MATERIALS AND METHODS

Yeast Two-Hybrid System. DNA encoding the entire cytoplasmic tail of NHE3 (amino acids 475–832) was cloned into the yeast LexA DNA-binding vector pEG202 (13). The resulting plasmid, pEG:C3, was used as a bait in two-hybrid screening of a WI-38 human lung fibroblast cDNA fused to the activation domain of GAL4 in the pJG4-5 plasmid. This cDNA library was kindly provided by Claude Sardet (Centre National de la Recherche Scientifique, Nice, France). To screen for interacting proteins, the yeast strain EGY48 was sequentially transfected with the bait plasmid and the cDNA library to obtain $\approx 3.5 \times 10^6$ primary transformants. Ninety-six of the primary transformants grew in the absence of leucine and had detectable β-galactosidase activity on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) plates. Positive library plas-

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0027-8424/97/943010-6$2.00/0
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Abbreviations: VSVG, vesicular stomatitis virus glycoprotein; MBP, maltose-binding protein; GST, glutathione *S*-transferase; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cAMP; PKA, cAMP-dependent protein kinase.

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mids were rescued from the yeast colonies and were transformed into EGY48 harboring a nonspecific bait, pRFHM-1, which is *lex*A DNA-binding domain fused to *Drosophila* bicoid protein in pEG202 to reconfirm the specificity of the interaction. Fifteen of the 96 initial positives interacted only with the pEG:C3 bait and not with the pRFHM-1 bait. These clones were sequenced and their sequences were compared with sequences in databanks using National Center for Biotechnology Information (NCBI) BLAST.

Expression of E3KARP and NHERF. To express E3KARP, the cDNAs from two partial clones, C16 and C42, were cloned into the mammalian glutathione *S*-transferase (GST) fusion protein vector, pBC (14). For transient expression of E3KARP, PS120/NHE3V fibroblasts were transfected with pBC constructs using lipofectin (GIBCO/BRL) and were grown for 48 hr before use. PS120/NHE3V fibroblasts are PS120 cells stably transfected with rabbit NHE3 tagged at its carboxyl terminus with an antibody epitope (YTDIEMNRLGK) derived from vesicular stomatitis virus glycoprotein (VSVG; refs. 15 and 16). For stable transfection in PS120/NHE3V fibroblasts, pBC constructs were cotransfected with pPlo2 (a gift of J. Gearhart at The Johns Hopkins University School of Medicine) for selection by hygromycin. To express NHERF, the 1.9-kb cDNA encoding NHERF was cloned into pECE and cotransfected with pPlo2 for selection by hygromycin. Cells resistant to 600 units/ml hygromycin were selected for eight passages before the *in vivo* binding and the functional analyses. Transfected PS120 fibroblasts were grown as previously published (5).

***In Vitro* and *in Vivo* Interaction.** Two partial clones of E3KARP, C16 and C42, were cloned into the maltose-binding protein (MBP) fusion protein vector pMAL-c2 vector (New England Biolabs). MBP and MBP fusion proteins were expressed in *E. coli*, and the recombinant proteins were purified by immobilization to amylose–agarose beads according to the manufacturer (New England Biolabs). To prepare cell extracts of PS120/NHE3V fibroblasts, the cells grown to confluence in a 10-cm Petri dish were lysed in N buffer (50 mM Hepes/Tris, pH 7.4/150 mM NaCl/3 mM KCl/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/1 mM aprotinin/1 mM pepstatin/1 mM iodoacetamide) supplemented with 1% Triton X-100, followed by centrifugation at 100,000 × *g* at 4°C for 20 min. The MBP fusion proteins (2 μg), bound to amylose–agarose beads, were incubated (in the final detergent concentration of 0.1% Triton X-100) with 1/10 of the PS120/NHE3V fibroblast lysate prepared as above for 1 hr at 4°C. After washing of the beads (three times in N buffer and three times in N buffer containing 0.1% Triton X-100), protein complexes bound to the amylose–agarose beads were separated on SDS/polyacrylamide gels and coprecipitated NHE3V was immunoblotted with anti-VSVG antibody (a gift of D. Louvard, Curie Institute, Paris; refs. 15 and 16).

For *in vivo* interactions between E3KARP and NHE3V, PS120/NHE3V fibroblasts expressing GST-16, GST-42, or GST control were lysed in N buffer containing 0.1% Triton X-100, and GST fusion proteins were precipitated by immobilization to glutathione–Sepharose beads. Following extensive washing of the beads, protein complexes bound to the glutathione–Sepharose beads were separated on SDS/polyacrylamide gels followed by immunoblotting with anti-VSVG antibody.

Northern Blot Analysis. Human multiple-tissue Northern blots (CLONTECH) were probed with cDNA probes, which correspond to amino acids 264–436 for E3KARP and amino acid 228-termination codon plus 409 bp of the untranslated 3′ end for NHERF. The cDNA probes were hybridized at 42°C in 5× standard saline phosphate/EDTA (SSPE), 10× Denhardt's solution, 2% SDS, 50% formamide, and 100 μg/ml salmon sperm DNA. Blots were washed at room temperature in 2× standard saline citrate (SSC)/0.5% SDS, followed by high stringency washes at 55°C in 0.1× SSC/0.1% SDS.

Western Blot Analysis. Western immunoblots were performed as described previously (11). Polyclonal antibody was prepared in rabbit against a synthetic peptide (KGPNGYGFNL) derived from the NHERF sequence (11).

Measurement of Na^+/H^+ Exchange. The Na^+/H^+ exchanger activities of stably transfected PS120 cells were studied fluorometrically using the pH-sensitive dye, 2′,7′-bis(2-carboxyethyl)-5,6 carboxyfluorescein (BCECF), and measurement of Na^+/H^+ exchange rates was done as described (5). All comparisons were done on cells of the same passage and number of acid loadings and studied on the same day to eliminate variability in the basal rate of Na^+/H^+ exchange as described (5). The values of V_{max}, $K'(H_i^+)$ (apparent affinity constant for intracellular H^+), and n_{app} (apparent Hill coefficient) generated above were used for statistical analyses (one-way ANOVA) to test whether or not there were differences between control and treated cells.

RESULTS

Nucleotide Sequence Analysis of E3KARP. Three (C16, C42, and C54) of the clones obtained by the two-hybrid screening encoded the same protein, which will be referred to as E3KARP (NHE3 kinase A regulatory protein). C42 and C54 were identical in nucleotide sequence encoding amino acids 130–268, and C16 extended from amino acid 9 to the 3′ poly(A) tail. C16 lacked the first eight amino acid residues at the amino terminus of E3KARP, since this was cloned as a fusion protein to the GAL4 activation domain. A search of protein sequence databases with BLAST revealed an identical sequence (GenBank accession no. Z50150) cloned by A. Ullrich's group and, therefore, the 5′ coding sequence was obtained by PCR based on the sequence in GenBank. The BLAST search also revealed an identical nucleotide fragment mapped at human chromosome 16p13.3 (17).

E3KARP encodes a protein of 451 aa with a calculated molecular weight of 50,000 (Fig. 1). In contrast, NHERF is composed of 353 aa with a deduced molecular weight of 39,000. E3KARP and NHERF share 44% identity, which is mostly concentrated within the first 260 aa. The carboxyl termini of the two proteins do not show any significant similarity. Based on the limited degree of identity, E3KARP does not appear to be a human homolog of NHERF, but it is a distinct protein. Whether these protein belong to the same gene family cannot be predicted based on the sequence homology. The first 260 aa of E3KARP contain two domains (amino acids 9–96 and 151–237), which show >70% identity between each other and between the equivalent domains of NHERF. The BLAST search repeatedly identified the PDZ (PSD-95, Dlg, and ZO-1) domains of the postsynaptic density protein, PSD-95, and *Drosophila* disc large protein with high degrees of homology ($P = 2 \times 10^{15} - 1 \times 10^{10}$). The PDZ domain is a module for protein–protein interaction (18, 19).

E3KARP Interacts with NHE3 *in Vitro* and *in Vivo*. To corroborate the results obtained with the two-hybrid system, the two clones of E3KARP, C16 and C42, were expressed as recombinant fusion proteins to MBP in *E. coli* (Fig. 1*Ba*) and purified by immobilization to amylose resins. The immobilized proteins were incubated with lysate from PS120/NHE3V fibroblasts. After extensive washing, bound NHE3V was analyzed by immunoblotting using anti-VSVG antibodies. As shown in Fig. 1*B*, NHE3V specifically bound to MBP-16 and MBP-42 but not to MBP control protein, confirming the interaction of NHE3 with E3KARP in yeast.

To test whether NHE3 is able to interact with C16 and C42 *in vivo*, we expressed C42 and C16 as GST fusion proteins. Initially, the pBC constructs were transiently transfected into the PS120/NHE3V fibroblasts, but the transient expression level of GST-16 did not yield any detectable amount of the protein and therefore a stable cell line was generated for GST-16. PS120/NHE3V/GST-16 was used for both the *in vivo*

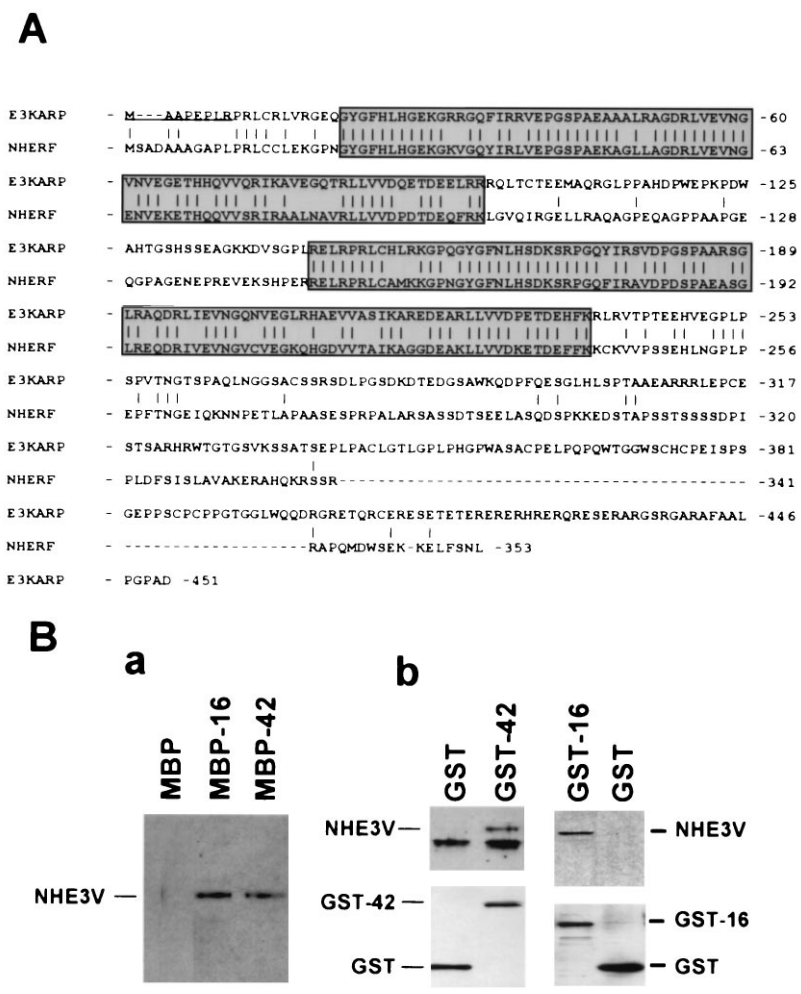

FIG. 1. (*A*) Alignment of the amino acid sequences of E3KARP and NHERF. Identical amino acid residues are indicated by |. The conserved PDZ domains are boxed. C42 and C54 were identical in size, encoding amino acids 130–268, and C16 extends from amino acid 9 to the 3′ poly(A) tail. The amino-terminal sequence of E3KARP (underlined) was obtained by PCR based on the sequence in GenBank. (*B*) *In vitro* and *in vivo* interaction of C16 and C42 with NHE3. (*a*) MBP-16, MBP-42, and MBP control proteins were immobilized on amylose–agarose beads and were incubated with detergent-solubilized lysates from PS120/NHE3V cells. Shown are immunoblots of NHE3V bound to MBP, MBP-16, and MBP-42. (*b*) PS120/NHE3V fibroblasts expressing GST, GST-42, or GST-16 were lysed and GST fusion proteins were purified on glutathione–Sepharose beads. Copurified NHE3V proteins were detected by Western immunoblot. (*Upper*) Immunoblots of copurified NHE3V. (*Lower*) Immunoblots of GST fusion proteins expressed in PS120/NHE3V cells. (*Left*) GST and GST-42 transiently transfected in PS120/NHE3V cells. (*Right*) GST-16 and GST stably transfected in PS120/NHE3V cells.

interaction study and the functional characterization of E3KARP. GST fusion proteins and GST control protein were purified by immobilization on glutathione–Sepharose beads and associated NHE3V was detected by Western immunoblotting using polyclonal anti-VSVG antibodies. Fig. 1*Bb* shows the NHE3V protein coprecipitated with GST-42 or GST-16 but not with GST control, demonstrating positive interaction between NHE3 and E3KARP in eukaryotic cells.

E3KARP and NHERF Are Not Expressed in PS120 and Caco-2 Cells. cAMP-mediated inhibition of the brush border Na^+/H^+ exchanger in ileal and renal tissues is well documented (1, 2, 4). However, when NHE3 was transfected into PS120 fibroblasts or the human colonic carcinoma cell line Caco-2, cAMP failed to inhibit NHE3 (5). We hypothesized that this lack of cAMP effect was due to the absence of regulatory proteins essential for the cAMP effect. To test this hypothesis, Northern blot analysis was performed to examine the expression of E3KARP and NHERF in PS120 fibroblasts, Caco-2 cells, and rabbit ileal villus cells, in which cAMP inhibits brush border Na^+/H^+ exchanger, as a control. Fig. 2*A* shows that rabbit ileum expresses 1.8- to 1.9-kb transcripts for both E3KARP and NHERF. In contrast, the transcripts were absent in PS120 and Caco-2 cells, consistent with the above hypothesis. The same size transcripts for both NHERF and E3KARP were unexpected, but the cDNA probes used for Northern hybridization correspond to the carboxyl termini of E3KARP and NHERF, where no significant homology exists. In addition, under the same conditions, the cDNA probes did not crosshybridize to the other cDNA (data not shown).

Correlation Between the cAMP Effect on NHE3 and the Expression of the Regulatory Proteins. We sought to determine whether the correlation between the cAMP effect on NHE3 and the expression of the regulatory proteins also exists in other tissues. We determined the expression of regulatory proteins by Western immunoblot using a polyclonal antibody against NHERF (11). As shown in Fig. 2*B*, an NHERF-related protein is expressed in OK, CHO, and LLC-PK_1 cells and rabbit kidney in all of which cAMP causes an acute inhibition of NHE3 activity (2, 11, 20–22). By contrast, an NHERF-related protein is absent in PS120 fibroblasts (Fig. 2*B*).

E3KARP and NHERF Mediate cAMP-Dependent Protein Kinase (PKA)-Dependent Inhibition of NHE3. To determine whether the regulatory proteins would reconstitute cAMP-induced inhibition of NHE3 activity, E3KARP and NHERF were expressed in PS120/NHE3V fibroblasts. In PS120/NHE3V/NHERF fibroblasts, treatment of the cells with 0.5 mM 8-bromoadenosine 3′,5′-cAMP (8-Br-cAMP) for 5–10 min before measurement of Na^+-dependent recovery resulted in ≈30% inhibition in NHE3 activity (Fig. 3*A*; tabulated in Table 1) by decreasing V_{max} from 2,206 ± 228 μM/sec for untreated to 1,579 ± 183 μM/sec for cAMP-treated cells, without any effect on the affinity for intracellular H^+ [$K'(H_i^+)$] or the Hill coefficient (n_{app}). The regulation of NHE3 activity by the V_{max} effect by cAMP is consistent with the previous studies that NHE3 is regulated by changes in V_{max} by second messengers, such as fetal bovine serum, epidermal growth factor, phorbol ester, and calmodulin (5). In contrast, 8-Br-cAMP had no effect on NHE3 activity in PS120/NHE3V fibroblasts (Fig. 3*B*).

E3KARP was expressed as GST-E3KARP (GST-16), which we demonstrated to interact with NHE3V (Fig. 1*B*). As with NHERF, GST-E3KARP expression resulted in ≈30% inhibition of NHE3 activity by decreasing V_{max} from 1,381 ± 188 μM/sec for untreated to 998 ± 164 μM/sec for cAMP-treated

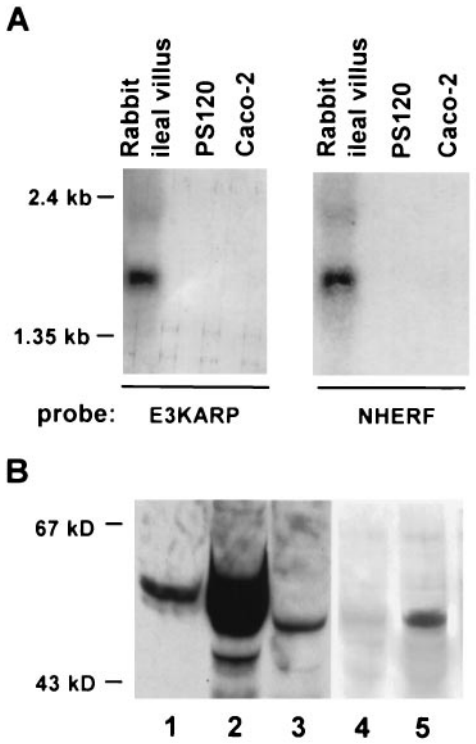

FIG. 2. (*A*) Northern blot analysis of E3KARP and NHERF expression in rabbit ileal villus cells, PS120, and Caco-2 cells. Two micrograms of poly(A)$^+$ enriched RNA from either PS120 fibroblasts or Caco-2 cells was electrophoresed per lane. For the rabbit ileal villus cells, 30 μg of total RNA was used. Hybridization was done under high-stringency conditions to avoid crosshybridization between E3KARP and NHERF. (*B*) Western blot analysis of expression of NHERF. Lanes: 1, brush border membrane from rabbit kidney and crude membranes from 2, CHO; 3, OK; 4, PS120; and 5, LLC-PK$_1$ cells. All lanes were loaded with 15 μg of membranes and were probed with polyclonal antibody against NHERF.

cells, with no effect on $K'(H_i^+)$ or n_{app}. In contrast, cAMP had no effect on PS120/NHE3V/GST control cells (Fig. 3*D*).

Human Tissue Distribution of E3KARP and NHERF mRNA. The presence of associated regulatory proteins for Na^+/H^+ exchangers have previously been predicted (9). However, finding two related proteins with the same function was unexpected. To examine relative expression of E3KARP and NHERF in various tissues, Northern blot analysis was performed on human tissues. Fig. 4 shows that the E3KARP transcript is expressed in most human tissues. E3KARP transcript is present in human colon, small intestine, ovary, prostate, spleen, heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, and, to a lesser extent, thymus. No transcript was detected in peripheral blood leukocytes. NHERF mRNA distribution is similar to that of E3KARP except that the NHERF transcript is absent from lung and skeletal muscle and present in a lesser amount in ovary. The expression of NHERF in human spleen, heart, and brain is in contrast to the absence in rabbit (12). In some tissues, additional transcripts of $\approx$2.3 and/or 1.45 kb were present, but the significance of these transcripts is not known.

DISCUSSION

Recent studies have shown that there is a discrepancy between protein kinase/growth factor-dependent regulation of NHE3 and the lack of changes in the phosphorylation level of NHE3. Therefore, in the absence of changes in phosphorylation level of NHE3, we hypothesized that there must be regulatory accessory proteins that interact with NHE3 in response to growth factor/protein kinase-mediated cellular signals. How the interaction with regulatory proteins affects Na^+/H^+ exchange activity is not known. Nor is it known whether phosphorylation of regulatory proteins is a prerequisite for the interaction with NHE3 to occur. Our screening of regulatory proteins using a yeast genetic system was performed in the absence of posttranslational modification by protein kinases due to the limitation of the two-hybrid system (23). That different sets of proteins might have been cloned in the presence of second messenger modulation cannot be overlooked. However, our preliminary data indicated that some proteins may bind to NHE3 in the absence of second messenger modulation (C.H.C.Y., unpublished data). In addition, Goss *et al.* (24) and Lin and Barber (25) identified proteins that interact with NHE1 in the absence of any modulation by protein kinases. Therefore, we made the assumption that some putative regulatory proteins can interact with Na^+/H^+ exchangers even in the absence of posttranslational modification and attempted to identify some of the regulatory proteins for NHE3.

In this study, we describe the initial results of a systematic screening for proteins that interact with NHE3. For this purpose, we have used a yeast genetic system for direct cloning of cDNAs encoding proteins that interact with a given target protein. We used the cytoplasmic tail of NHE3 in a LexA fusion expression plasmid as the bait for cloning since our previous studies have shown the essential role of the carboxyl-terminal tail in NHE3 regulation (26).

Three of the clones obtained encoded the same protein, E3KARP. E3KARP shows a high degree of similarity with NHERF (12). The alignment of the deduced amino acid sequences shows that there are two regions that are highly conserved between the two proteins: amino acids 9–96 and 151–237 of E3KARP. These two regions, named PDZI (amino acids 9–96) and PDZII (amino acids 151–237), share >70% identity between each other and between the two proteins. These regions show a significant identity (48–53%) to a motif known as a PDZ sequence motif (27). Recent studies demonstrated that PDZ domains play a major role in protein–protein interaction, assembling components involved in cellular signaling at the plasma membrane (18, 19).

In light of these recent findings on the PDZ domains, the presence of two PDZ domains in both regulatory proteins was intriguing. In this present work, we have not specifically defined domains within E3KARP necessary for the interaction with NHE3, but the interaction of C42, which is mostly composed of PDZII, with NHE3 *in vivo* and *in vitro* suggests that the PDZ domain probably contributes to the interaction between NHE3 and E3KARP. A number of studies have shown that the PDZ-binding sequence consists of a S/TXV motif at the carboxyl terminus (18, 19). NHE3 lacks the S/TXV motif at the carboxyl terminus and the tagging of the VSVG epitope at the carboxyl terminus of NHE3 (NHE3V) did not interfere with the binding of NHE3 to E3KARP (Fig. 2*B*). This suggests that the interaction does not involve the carboxyl terminus of NHE3 but occurs via an internal site yet to be identified. An internal S/TXV motif has been shown to be involved in binding to the PDZ domain of another protein (28).

The ileal and renal brush border Na^+/H^+ exchangers are characteristically inhibited by cAMP. However, when NHE3 as well as NHE1 and NHE2 were transfected into PS120 fibroblasts or Caco-2 cells, cAMP inhibition was not observed (5). This absence of the cAMP effect is due neither to the inability of PS120 fibroblasts and Caco-2 cells to respond to cAMP nor to the inability of NHE3 to respond to cAMP. This is based on the following facts: (*i*) β-NHE, a Na^+/H^+ exchanger isolated from trout red blood cells, is stimulated by cAMP when expressed in PS120 fibroblasts (29); (*ii*) Caco-2 cells can respond to cAMP-induced cellular signals, since cAMP causes Cl^- secretion in these cells (30–32); and (*iii*) the rat NHE3, when expressed in the Na^+/H^+ exchange-deficient Chinese hamster ovary fibroblast (AP-1), is down-regulated by 8-Br-cAMP (21, 22). Therefore, we postulated that the lack of the

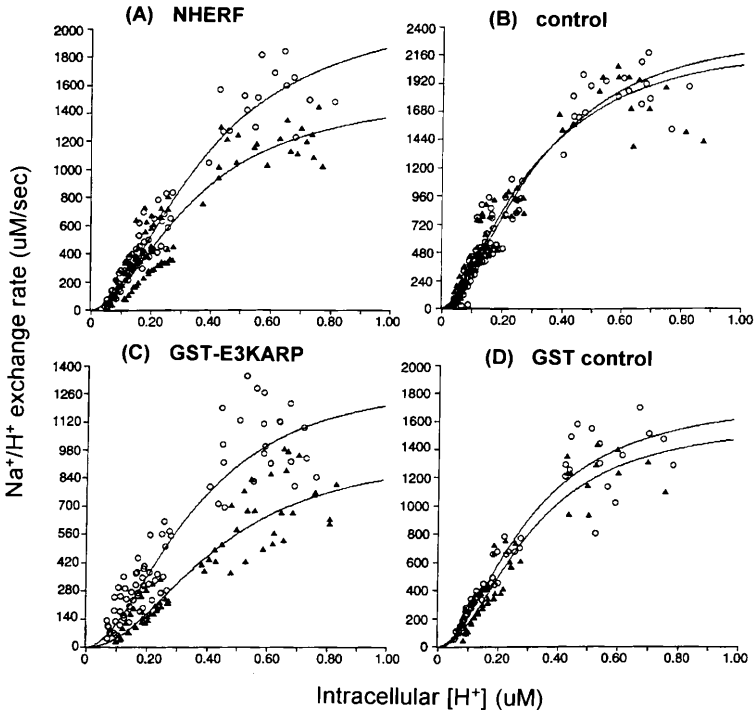

FIG. 3. cAMP-induced inhibition of NHE3. Stably transfected PS120 fibroblasts acidified with NH_4Cl were either recovered in Na^+ medium (○) or treated with 0.5 mM 8-Br-cAMP for 5–10 min before the recovery in Na^+ medium (▲). Na^+/H^+ efflux rates were calculated at various pH_i, and lines were fitted to the data using an allosteric model. Treatment of (*A*) PS120/NHE3V/NHERF with 0.5 mM 8-Br-cAMP (▲) inhibited the Na^+/H^+ exchange activity with a decrease in V_{max} by ≈30% with no effect on $K'(H_i^+)$ or n_{app}. (*C*) Similarly, PS120/NHE3V/GST-E3KARP was inhibited by 8-Br-cAMP by a decrease in V_{max} by ≈28%. In contrast, PS120/NHE3 (*B*) and PS120/NHE3/GST (*D*) were not affected by 8-Br-cAMP. Shown here are data from four or more experiments for each condition.

cAMP effect on NHE3 in PS120 and Caco-2 cells is due to the absence or underexpression of a protein(s) that modulates NHE3 activity in response to cAMP in these cells. In accordance with our hypothesis, neither PS120 fibroblasts nor Caco-2 cells express E3KARP or NHERF. We also showed that other cell lines including AP-1, OK, and LLC-PK_1 cells, in which NHE3 inhibition by cAMP was demonstrated, expressed NHERF-related proteins. Therefore, there appears to be a correlation between the cAMP elicited inhibition of NHE3 and the expression of the regulatory proteins. Since the antibody used for the Western blot (Fig. 2*B*) was raised against a peptide sequence (amino acids 158–172 of NHERF), which is highly conserved in both NHERF and E3KARP, the antibody crossreacted with both NHERF and E3KARP (C.H.C.Y., unpublished data). Thus it cannot be determined by Western analysis whether one or both regulatory proteins are expressed in these cell lines.

The first indication of the presence of a regulatory protein for PKA-dependent inhibition of NHE3 came from Weinman *et al.* (33), who used limited trypsin digestion of BBM proteins to show that the activity of the BBM Na^+/H^+ exchanger could be dissociated from its inhibition by PKA. Subsequent fractionation by column chromatography identified a 42- to 44-kDa protein in renal brush border membrane vesicles (11). The cDNA encoding NHERF has recently been isolated (12), but a direct effect on cAMP-induced inhibition of NHE3 had not been demonstrated until the present work. The expression of either NHERF or E3KARP in PS120/NHE3 fibroblasts, which lack any endogenous expression of these regulatory proteins, reconstituted the PKA-induced inhibition of NHE3. Either protein resulted in ≈30% inhibition by PKA. The extent of inhibition transduced by each regulatory protein is comparable to the magnitude of cAMP-dependent inhibition reported in brush border vesicles (34). It is not known whether the two regulatory protein show an additive effect. Neither is known whether E3KARP and NHERF transduce cAMP effect specifically on NHE3 or can reconstitute the cAMP-induced regulation of NHE1 and NHE2. Of note, NHE1 did not show any interaction with C16 or C42 by the two-hybrid system (C.H.C.Y., unpublished data), suggesting that NHE1 is stimulated by PKA via a different route than NHE3.

Human tissue distributions of E3KARP and NHERF transcripts are remarkably similar, with the exception of lung and skeletal muscle lacking the NHERF transcript. Both NHERF and E3KARP transcripts are expressed in human intestine and kidney, where NHE3 functions in Na^+ absorption. E3KARP and NHERF transcripts are also expressed in other human tissues, including thymus, prostate, testis, ovary, spleen, brain, and placenta, all of which also have human NHE3 transcripts (35). However, the expression of NHERF and/or E3KARP transcripts are not limited to tissues expressing NHE3. Heart, lung, liver, skeletal muscle, and pancreas do not appear to

Table 1. Effects of cAMP on NHE3 activity in PS120/NHE3V fibroblasts transfected with NHERF or E3KARP

Cell lines	Treatment	V_{max}, μM/sec	$K'(H_i^+)$, μM	n_{app}
PS120/NHE3V/NHERF	Control	2,206 ± 223	0.18 ± 0.07	1.8
	+ 8-Br-cAMP	1,579 ± 183*	0.15 ± 0.07	1.8
PS120/NHE3V	Control	2,401 ± 176	0.12 ± 0.05	1.7
	+ 8-Br-cAMP	2,283 ± 170	0.12 ± 0.04	1.7
PS120/NHE3V/GST-E3KARP	Control	1,382 ± 180	0.14 ± 0.08	1.8
	+ 8-Br-cAMP	998 ± 164*	0.17 ± 0.07	2.1
PS120/NHE3V/GST	Control	1,796 ± 124	0.11 ± 0.04	1.8
	+ 8-Br-cAMP	1,612 ± 172	0.10 ± 0.05	2.0

Data are presented as means ± SE. $n \geq 4$ for all experiments. Na^+/H^+ exchange activity measurements shown in Fig. 3 are tabulated.
*$P < 0.05$ vs. control.

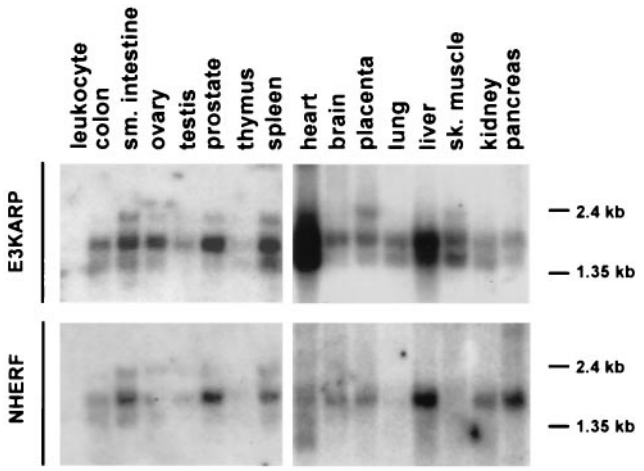

FIG. 4. Expression of E3KARP and NHERF mRNAs in human tissues. E3KARP and NHERF cDNAs that do not crosshybridize were used as probes. Both blots (*Left*, MTN blot II; *Right*, MTN blot) were commercially prepared and purchased from CLONTECH. Northern blot analyses were performed at four different times under the same stringency conditions and therefore cannot be used to compare the relative intensity between the blots.

express NHE3 transcripts but do contain the transcripts of E3KARP or NHERF, suggesting that these proteins may serve functions unrelated to Na^+ absorption by NHE3. Interestingly, the same cDNA (named TKA-1) was cloned by Alex Ullrich's group, and TKA-1 was preliminarily implicated in interaction with the platelet-derived growth factor receptor (GenBank accession no. Z50150).

Recent work by Moe *et al.* (21) have shown that the acute inhibition of NHE3 by PKA is accompanied by an increase in phosphocontent of NHE3 expressed in AP-1 cells. However, as suggested by the authors, this indicates a potential role of phosphorylation in NHE3 inhibition, but the necessity of the NHE3 phosphorylation by PKA for the inhibition requires further investigation. Based on the sequence homology, both E3KARP and NHERF appear not to be kinases or phosphatases, suggesting that direct phosphorylation of NHE3 by these regulatory proteins is unlikely. The deduced amino acid sequences of both E3KARP and NHERF lack the classical PKA phosphorylation motif, R-R/K-X-S (10), although NHERF, but not E3KARP, has an alternative PKA consensus phosphorylation site at amino acid 340 (KRSS; ref. 12) and is reported to be a phosphoprotein (34). Despite the lack of the consensus phosphorylation site, recombinant E3KARP and NHERF prepared from *E. coli* are substrates for phosphorylation by PKA *in vitro* (G. Lamprecht and C.H.C.Y., unpublished data). Thus far, how the signal is transduced from PKA to NHE3 is not known. In light of the findings by Moe *et al.* (21), the direct phosphorylation of NHE3 by PKA seems appealing. In this situation, the regulatory proteins may function by an as yet unknown mechanism to allow PKA to access NHE3. However, the possibility of NHE3 inhibition by more than one mechanism, one via phosphorylation of NHE3 and the other via phosphorylation of the regulatory proteins, cannot be ruled out.

Our present studies show that PKA-dependent inhibition of NHE3 requires a regulatory protein to mediate the cellular signal. Either E3KARP or NHERF is necessary for the cAMP-dependent inhibition of NHE3. The presence of both E3KARP and NHERF in intestine and kidney suggests a potential interaction between the two regulatory proteins. To our knowledge, this is the first demonstration that proteins that contain PDZ domains are directly involved in signal transduction. However, the role of the PDZ domains in interaction with NHE3 or other regulatory proteins needs to be further investigated.

The WI-38 human lung fibroblast library was kindly provided by Dr. Claude Sardet at Centre National de la Recherche Scientifique (Nice, France). Plasmid pBC was kindly provided by Drs. B. Chatton and C. Kedinger at Laboratorie de Génétique Moleculaire des Eucaryotes/CNRS (Strasbourg, France). We thank Drs. J. Handler and G. Breitweiser at The Johns Hopkins University School of Medicine for useful discussions. This work was supported in part by American Digestive Health Foundation/American Gastroenterological Association/Hoechst Marion Rousel Research Scholar Award, an Institutional Research Grant from The Johns Hopkins University, National Institutes of Health (NIH) Grants DK08950, DK 26523, DK44484, and DK37316, Fogarty International Center NIH TW05234, and funds from Research Service, Department of Veterans Affairs and the Meyerhoff Digestive Diseases Center.

1. Donowitz, M. & Welsh, M. J. (1986) *Annu. Rev. Physiol.* **48,** 135–150.
2. Weinman, E. J. & Shnolikar, S. (1993) *Annu. Rev. Physiol.* **55,** 289–304.
3. Yun, C. H. C., Tse, C.-M., Nath, S. K., Levine, S. A., Brant, S. R. & Donowitz, M. (1995) *Am. J. Physiol.* **269,** G1–G11.
4. Aronson, P. S. & Igarashi, P. (1986) *Curr. Top. Membr. Transp.* **26,** 57–75.
5. Levine, S. A., Montrose, M. H., Tse, C. M. & Donowitz, M. (1993) *J. Biol. Chem.* **268,** 25527–25535.
6. Goss, G. G., Woodside, M., Wakabayashi, S., Pouyssegur, J., Waddel, T., Downey, G. P. & Grinstein, S. (1994) *J. Biol. Chem.* **269,** 8741–8748.
7. Demaurex, N. & Grinstein, S. (1994) *J. Exp. Biol.* **196,** 389–404.
8. Cabado, A. G., Yu, F. H., Kapus, A., Lukacs, G., Grinstein, S. & Orlowski, J. (1996) *J. Biol. Chem.* **271,** 3590–3599.
9. Wakabayashi, S., Bertrand, B., Shigekawa, M., Fafournoux, P. & Pouyssegur, J. (1994) *J. Biol. Chem.* **269,** 5583–5587.
10. Adams, J. A. & Taylor, S. S. (1993) *J. Biol. Chem.* **268,** 7747–7752.
11. Weinman, E. J., Steplock, D. & Shenolikar, S. (1993) *J. Clin. Invest.* **92,** 1781–1786.
12. Weinman, E. J., Steplock, D., Wang, Y. & Shenolikar, S. (1995) *J. Clin. Invest.* **95,** 2143–2149.
13. Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. (1993) *Cell* **75,** 791–803.
14. Chatton, B., Bahr, A., Acker, J. & Kedinger, C. (1995) *BioTechniques* **18,** 142–145.
15. Kreis, T. E. (1986) *EMBO J.* **5,** 931–941.
16. Hoogerwerf, W. A., Tsao, S. C., Devuyst, O., Levine, S., Yun, C. H. C., Yip, J. W., Cohen, M., Wilson, P. D., Lazenby, A. J., Tse, M. & Donowitz, M. (1996) *Am. J. Physiol.* **270,** G29–G41.
17. Burn, T. C., Connors, T. D., Dackowski, W. R., VanRaay, T. J., Millholland, J. M., Klinger, K. W. & Landes, G. M. (1996) *Genome Res.* **6,** 525–537.
18. Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N. & Sheng, M. (1995) *Nature (London)* **378,** 85–88.
19. Kornau, H.-C., Schenker, L. T., Kennedy, M. B. & Seeburg, P. H. (1995) *Science* **269,** 1737–1740.
20. Helme-Kolb, C., Montrose, M. H. & Murer, H. (1990) *Pflügers Arch.* **416,** 615–623.
21. Moe, O. W., Amemiya, M. & Yamaji, Y. (1995) *J. Clin. Invest.* **96,** 2187–2194.
22. Kandasamy, R. A., Yu, F. H., Harris, R., Boucher, A., Hanrahan, J. W. & Orlowski, J. (1995) *J. Biol. Chem* **270,** 29209–29216.
23. Phizicky, E. M. & Fields, S. (1995) *Microbiol. Rev.* **59,** 94–123.
24. Goss, G., Orlowski, J. & Grinstein, S. (1996) *Am. J. Physiol.* **270,** C1493–C1502.
25. Lin, X. & Barber, D. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 12631–12636.
26. Levine, S. A., Nath, S., Yun, C. H. C., Yip, J. W., Montrose, M. H., Donowitz, M. & Tse, C. M. (1995) *J. Biol. Chem.* **270,** 13716–13725.
27. Ponting, C. P. & Phillips, C. (1995) *Trends Biochem. Sci.* **20,** 102–103.
28. Shieh, B.-H. & Zhu, M.-Y. (1996) *Neuron* **16,** 991–998.
29. Borgese, F., Sardet, C., Cappadoro, M., Pouyssegur, J. & Mortais, R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6765–6769.
30. Huber, S., Koltgen, D., Roscher, A. A. & Heinz-Erian, P. (1993) *Pflügers Arch.* **424,** 364–366.
31. Tien, X. Y., Brasitus, T. A., Kaetzel, M. A., Dedman, J. R. & Nelson, D. J. (1994) *J. Biol. Chem.* **269,** 51–54.
32. Watson, A. M., Levine, S., Donowitz, M. & Montrose, M. H. (1991) *Am. J. Physiol.* **261,** G229–G238.
33. Weinman, E. J., Dubinsky, W. P., Dinh, Q., Steplock, D. & Shenolikar, S. (1989) *J. Membr. Biol.* **109,** 233–241.
34. Weinman, E. J., Steplock, D., Yuan, B. N. & Shenolikar, S. (1990) *Am. J. Physiol.* **158,** F1254–F1258.
35. Brant, S. R., Yun, C. H. C., Donowitz, M. & Tse, C.-M. (1995) *Am. J. Physiol.* **269,** C198–C206.