Cyclic AMP is a major second messenger that inhibits the brush border Na\(^+\)/H\(^+\) exchanger NHE3. We have previously shown that either of two related regulatory proteins, E3KARP or NHERF, is necessary for the cAMP-dependent inhibition of NHE3. In the present study, we characterized the interaction between NHE3 and E3KARP using \textit{in vitro} binding assays. We found that NHE3 directly binds to E3KARP and that the entirety of the second PSD-95/DigZO-1 (PDZ) domain plus the carboxyl-terminal domain of E3KARP are required to bind NHE3. E3KARP binds an internal region within the NHE3 C-terminal cytoplasmic tail, defining a new mode of PDZ domain interaction. Analyses of cellular distribution of NHE3 and E3KARP expressed in PS120 fibroblasts show that NHE3 and E3KARP are co-localized on the plasma membrane, but not in a distinct juxtanuclear compartment in which NHE3 is predominantly expressed. The distributions of NHE3 and E3KARP were not affected by treatment with 8-bromo-cAMP. As shown earlier for the human homolog of NHERF, we also found that the cytoskeletal protein ezrin binds to the carboxy-terminal domain of E3KARP. These results are consistent with the possibility that E3KARP and NHERF may function as scaffold proteins that bind to both NHE3 and ezrin. Since ezrin is a protein kinase A anchoring protein, we suggest that the scaffolding function of E3KARP binding to both ezrin and NHE3 localizes cAMP-dependent protein kinase in the vicinity of the cytoplasmic domain of NHE3, which is phosphorylated by elevated cAMP.

NHE3 is the Na\(^+\)/H\(^+\) exchanger in the brush border membrane of the proximal tubule and small intestine and colon that plays a major role in transepithelial Na\(^+\) absorption (1, 2). Among the various stimuli that modulate NHE3 activity (1), cAMP inhibits the transepithelial Na\(^+\) absorption by NHE3. However, the molecular mechanisms of the cAMP-dependent inhibition of the Na\(^+\) absorptive process remain unknown.

Currently, two major mechanisms have been suggested for the cAMP-dependent inhibition of NHE3 activity. The first involves an increase in NHE3 phosphorylation level by PKA,\(^1\) and the second requires the presence of a regulatory factor. NHE3 phosphorylation by PKA was initially demonstrated by Moe \textit{et al.} (3), who showed that in AP-1 cells, a Chinese hamster ovary cell line, the cAMP-elicited inhibition of NHE3 activity was accompanied by an increase in NHE3 phosphorylation level. More recently, Kurashima \textit{et al.} (4) identified the sites of phosphorylation in NHE3 by PKA in AP-1 cells. In contrast, we previously reconstituted the cAMP-dependent inhibition of NHE3 by transfecting one of two regulatory proteins, E3KARP (NHE3 kinase A regulatory protein\(^2\); also named TKA-1) or NHERF (NHE regulator factor), in the PS120 fibroblast cell line, which lacks these regulatory proteins, and consequently, cAMP does not affect NHE3 activity (5–7). This demonstrates the necessity of a regulatory protein in cAMP-dependent inhibition of NHE3. E3KARP was cloned from a human lung library by yeast two-hybrid screening using the cytoplasmic domain of NHE3 as bait (5). NHERF was identified by limited trypsinization and cellular fractionation of rabbit renal brush border vesicles (6, 8). E3KARP and NHERF are closely related proteins of 337 and 358 aa, respectively. These proteins share 52% identity, and both contain two tandem protein interaction module PDZ domains (5). How these regulatory proteins mediate the inhibitory effect of cAMP is not clear.

Protein-protein interactions are intrinsic to virtually every cellular process. Many of these interactions are mediated by modular domains such as the Src homology SH2 and SH3, pleckstrin homology, and PDZ domains (9–12). We have shown previously that NHE3 and E3KARP directly interact with each other (5). In this present report, we analyzed the interaction between NHE3 and E3KARP to determine minimal domains necessary for the interaction. We found that the interaction of NHE3 with E3KARP represents a new mode of PDZ binding and that it requires a PDZ domain and a non-PDZ domain of E3KARP. We also found that E3KARP binds to an internal domain within the NHE3 cytoplasmic tail, which has previously been shown to be essential for PKA-dependent inhibition of NHE3 (13). In addition, we show that E3KARP binds to the cytoskeletal protein ezrin, which was previously shown to bind to PKA. These findings suggest a potential model for E3KARP in the inhibition of NHE3 activity by PKA.

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\(^1\) The abbreviations used are: PKA, cAMP-dependent protein kinase; aa, amino acid(s); PCR, polymerase chain reaction; NTA, nitroliotriacetic acid; MBP, maltose-binding protein; N-ezrin, N-terminal 302 aa of human ezrin; 8-Br-cAMP, 8-bromoadenosine 3\'-5\'-monophosphate; PBS, phosphate-buffered saline; PDZ, PSD-95/DigZO-1.

\(^2\) The corrected sequence is found under accession number AF004900 in the GenBank\(^\text{TM}\) Data Bank.
E3KARP and domains of E3KARP were expressed as His6-tagged and/or MBP fusion proteins. The italic numbers indicate the amino acids at the start and the end of each domain. C42 is a partial clone of E3KARP obtained by yeast two-hybrid screening (5).

EXPERIMENTAL PROCEDURES

Cell Culture—PS120 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 25 mM NaHCO3, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 1% fetal bovine serum in a 5% CO2, 95% air incubator at 37 °C.

Gel Overlay Assay—Various domains of E3KARP were generated by PCR and were expressed as recombinant proteins in E. coli. These domains include the first PDZ domain (P1) and the second PDZ domain (P2), expressed individually (P1 or P2) or together as a unit (P1–P2); the C-terminal domain (C); and the second PDZ domain and the C-terminal domain as a single unit (P2–C) (Fig. 1). We also expressed clone 42 (C42), which extends from aa 130 to 314 of E3KARP and was one of the partial clones initially obtained from yeast two-hybrid screening (5). Fidelity of the PCR products was confirmed by nucleotide sequencing. The PCR products were cloned into pET30a (Novagen), and expression of NHE3 was selected by acid-precipitable and transfected into E. coli. Nitrocellulose membranes were washed four times with 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.2% Triton X-100, followed by boiling in SDS sample buffer. The samples were resolved by SDS-polyacrylamide gel electrophoresis and were immunoblotted with the mPD4 antibody.

Immunofluorescence—PS120 fibroblasts were grown on tissue culture coverslips. Cells were serum-starved overnight, and when necessary, cells were treated with 0.1 mM 8-bromoadenosine 3′,5′-monophosphate (8-Br-cAMP) for 20 min before washing with PBS. After washing with PBS, cells were fixed at room temperature for 10 min with 3% paraformaldehyde in PBS, followed by permeabilization with 0.2% Triton X-100 and 1% goat serum albumin, and 0.1% Tween 20 and mounted with Prolong Antifade (Molecular Probes, Inc.). Nuclei were stained with Hoechst 33342 (Molecular Probes, Inc.). Cells were examined with an inverted microscope (Zeiss Axiovert) coupled to a confocal laser scanning unit (Bio-Rad MRC-600).

Measurement of Na+/H+ Exchange—The Na+/H+ exchanger activities of stably transfected PS120 cells were measured using the ratio fluorometric pH-sensitive dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein as described previously (15). When 8-Br-cAMP was used, cells were pretreated with 100 μM 8-Br-cAMP for 15 min during the dye loading and during the prepulse period.

RESULTS

In Vivo Interaction between NHE3 and the Regulatory Factors—To demonstrate that E3KARP and NHE3 interact in vivo, we stably expressed E3KARP as a His6- and S-protein-tagged fusion protein, E3KARP-HS, in PS120/NHE3V fibroblasts. To ensure that the tagging of E3KARP protein with His6 and S-protein did not alter its function in cAMP-dependent inhibition of NHE3, we studied the activity of NHE3V in response to 100 μM 8-Br-cAMP. Fig. 2 shows that the sodium-dependent alkaline activation due to NHE3 activity is significantly inhibited in the presence of 8-Br-cAMP in PS120/NHE3V and E3KARP-HS cells. The mean intracellular pH value of PS120/NHE3V/E3KARP-HS in the absence or presence of 8-Br-cAMP was 7.38 ± 0.036 and 7.26 ± 0.027, respectively (p < 0.05 by unpaired t test; n = 5).

We then determined if there was any interaction between NHE3V and E3KARP-HS in vivo. The antibody against the C-terminal 106 aa of E3KARP, Ab2570, recognized E3KARP-HS expressed in PS120/NHE3V fibroblasts (Fig. 3A).
Western immunoblot using Ab2570. Thirty mE3KARP-HS fibroblasts were acidified with a NH4Cl prepulse, followed by perfusion with TMA buffer (15). Cells were then recovered either in Na+ buffer (broken line) or in Na+ buffer containing 0.1 mM 8-Br-cAMP (solid line). At the end of each experiment, the fluorescence ratio was calibrated using high potassium/nigericin buffer titrated at pH 6.1 and 7.2. Representative traces from five sets of experiments are shown.

The C-terminal 106 aa of E3KARP used in preparation of Ab2570 show limited homology to the similar C-terminal domain of NHERF (5). Therefore, Ab2570 exhibited minimal cross-reactivity with NHERF (Fig. 3A). E3KARP-HS was immunoprecipitated with Ab2570 against E3KARP, and the success of immunoprecipitation was detected with S-protein-horseradish peroxidase conjugate (Fig. 3B). Coprecipitated NHE3V was then detected with mouse monoclonal antibody mP5D4 against the VSVG epitope tag. As shown in Fig. 3B, NHE3V specifically co-immunoprecipitated with E3KARP-HS, demonstrating the interaction in vivo between NHE3 and E3KARP, consistent with our earlier finding (5).

E3KARP Binds NHE3 and Ezrin

E3KARP Binding Requires Both the Second PDZ Domain and the Carboxyl-terminal Tail of E3KARP. A, domains of E3KARP were expressed as His6-tagged fusion proteins and purified. Three μg of each sample was run on SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. The membrane was overlaid with 35S-labeled NHE3C as described under “Experimental Procedures.” Molecular mass standards (in kilodaltons) are indicated on the right. B, lysate prepared from PS120/NHE3V fibroblasts was incubated with His6-tagged recombinant E3KARP or domains of E3KARP immobilized on Ni2+-NTA beads with or without Ni2+-NTA beads alone, and bound proteins were resolved by SDS-polyacrylamide gel electrophoresis. Bound NHE3V was identified with the mP5D4 antibody.
E3KARP Binds to an Internal Domain of the NHE3 C Terminus—We next determined which part of the NHE3 C terminus interacts with E3KARP. Gel overlay assays were performed on E3KARP using the 35S-labeled truncated NHE3 C-terminal constructs. As shown in Fig. 6, the entire NHE3 C terminus (C3-832) and C3-711, which ranges from aa 475 to 711, bound to E3KARP. Similarly, C3-660, corresponding to aa 475–660, bound to E3KARP, although the binding of C3-660 to E3KARP was always significantly lower than that of C3-711.

We next determined which part of the NHE3 C terminus contributes to the interaction of E3KARP. This lack of binding of the C-terminal 88 aa of NHE3 to the regulatory proteins is consistent with the fact that NHERF interact with NHE3 and seemingly have the same binding specificity as E3KARP. The P2-C domain effectively blocked NHE3C binding to E3KARP and the P2-C domain. In contrast, the P1 domain showed no effect on the binding, consistent with the weak binding observed in the gel overlays.

E3KARP Binds NHE3 and Ezrin—Recent studies by Reczek et al. (19) and Murthy et al. (20) have shown that the human homolog of NHERF, EBP50, binds to the ezrin-radixin-moesin family. Since both E3KARP and NHERF interact with NHE3 and seemingly have the same function in the cAMP-dependent inhibition of NHE3, we determined if ezrin can also interact with E3KARP. The N-ezrin was labeled with [35S]methionine by in vitro transcription-translation and was used as a probe for gel overlay. NHERF (5, 6) expressed as a His6-tagged fusion protein was used as a positive control for the N-ezrin binding. Fig. 8A shows that the N-ezrin bound to MBP/E3KARP, but not MBP itself, indicating specific interaction between the N terminus of ezrin and E3KARP. The interaction of the N-ezrin with E3KARP was as strong as that with NHERF. To determine which part of E3KARP binds to the N-ezrin, interaction between the N-ezrin and the subdomains of E3KARP expressed as His6-tagged fusion proteins was studied by gel overlay assay. As shown in Fig. 8B, the N-ezrin did not bind to the individual PDZ domains (P1 and P2) or to P1–2 as one unit. However, the N-ezrin bound to the C-terminal and P2-C domains of E3KARP. Interestingly,
The N-ezrin did not interact with the C42 construct, which is essentially P2-C lacking the carboxyl-terminal 23 aa residues. The lack of N-ezrin binding to the C42 construct suggests that the ezrin-binding motif is located within the carboxyl-terminal 23 aa residues of E3KARP (Fig. 8C). Fig. 8D shows the distribution of E3KARP and ezrin in PS120/NHE3V/E3KARP-HS cells. Although both proteins were largely expressed in the cytoplasm as expected, some membrane staining was also evident. Double staining of endogenous ezrin and exogenous E3KARP revealed that ezrin and E3KARP are co-localized in some areas along the plasma membrane. Treatment with 8-Br-cAMP for 20 min did not alter the distribution of E3KARP or ezrin or the co-localization of these two proteins.

**DISCUSSION**

In this study, we determined the interaction between NHE3 and E3KARP. We specifically wanted to address whether the PDZ domains in E3KARP contribute to the interaction with NHE3. This was not only because PDZ domains were recently shown to be the basis for protein-protein interaction, but also because one of the clones obtained in our initial two-hybrid screen encompassed the second PDZ domain and a large part of...
**Fig. 8. Interaction of the N-terminal domain of ezrin with E3KARP.**

A. 3 μg each of bacterially expressed MBP/E3KARP, MBP, and NHERF-His<sub>6</sub> was overlaid with the 35S-labeled N-terminal 302 aa of human ezrin as described under “Experimental Procedures.”

B. His<sub>6</sub>-tagged E3KARP and domains of E3KARP were overlaid with the 35S-labeled N-terminal 302 aa of human ezrin.

C. The amino acid sequences of the C-terminal 30 aa of E3KARP and NHERF are aligned. Identical amino acid residues are indicated by vertical lines.

D. Shown is the co-localization of E3KARP and ezrin in PS120/NHE3V/E3KARP-HS fibroblasts. Immunofluorescence staining was performed on serum-starved cells under basal conditions (panels a–c) or after a 15-min incubation with 100 μM 8-Br-cAMP (panels d–f). E3KARP (panels a and d) was stained as described in the legend to Fig. 6. Ezrin (panels b and e) was stained with mouse monoclonal antibody 3C12, followed by Cy5-labeled anti-mouse antibody. Images were collected with ×40 objectives. E3KARP and ezrin are observed to co-localize on the plasma membrane (panels c and f).
the C terminus of E3KARP (5). Based on this, we anticipated that NHE3 might interact with E3KARP through the second PDZ domain.

Studies on protein-protein interaction via PDZ domains showed that distinct PDZ domains interact with specific carboxyl termini with a consensus sequence of E(S/T)X(V/I) (11, 12). However, certain PDZ domains can bind other PDZ domains. For example, the PDZ domain of nNOS can bind to the second PDZ domain of α-synaptophin in skeletal muscle (21). Interaction of E3KARP with NHE3 represents a new mode of binding for a PDZ domain. In this study, we found that the second PDZ domain was a major constituent in the interaction with the NHE3 C terminus, but the second PDZ domain alone was not sufficient for the interaction. Instead, the P2 domain and the C terminus of E3KARP formed a unit that interacted with NHE3. This is not the first case where a PDZ domain does not work in isolation. In other cases, paired PDZ domains were required for interaction (22, 23). Band 4.1 binds to the first and second PDZ domains of hDlg, a human homolog of the Drosophila tumor suppressor, as a unit, but not to individual PDZ domains (22). Similarly, binding of syndecan, a major cell-surface heparan sulfate, to the PDZ domain-containing protein syntenin requires two PDZ domains of syntenin (23). However, in this study, the P1–2 domain and also the P1 domain of E3KARP displayed weak binding characteristics that were not observed consistently, and we thus dismissed this as a possible artifact of the in vitro conditions. The interaction of the NHE3 C terminus with E3KARP differs from previously defined cases in that it required the second PDZ domain and a non-PDZ region, the C-terminal portion. Why the second PDZ domain of E3KARP does not work in isolation is not clear.

The P2-C domain of E3KARP is shown to interact with an internal domain of NHE3 between aa 585 and 660 of the NHE3 C-terminal tail. Since the relative E3KARP binding to C3-660 was significantly lower than that to C3-711, it is conceivable that the domain between aa 660 and 711 may also take part in binding to E3KARP. Concerning the mapping of the E3KARP-binding domain within the NHE3 C terminus, our analysis was limited to the gel overlay assays since we were not able to bind E3KARP to E3KARP. The mechanistic nature of the PKA-dependent regulation of NHE3 by cAMP.3 The lack of redistribution of E3KARP (and NHERF) is perhaps consistent with the fact that the phosphorylation level of neither protein was changed in response to 8-Br-cAMP.4 This study showed that NHE3N and E3KARP are co-localized at the plasma membrane, consistent with the interaction between NHE3N and E3KARP in vivo and the E3KARP-dependent regulation of NHE3 by cAMP. However, the distribution of NHE3N in PS120 fibroblasts is largely distinct from that of E3KARP, with NHE3N localized at perinuclear regions and on the surface membrane. The perinuclear staining is thought to represent NHE3 proteins present in recycling endosomes. Distribution of NHE3N was not changed by the presence of 8-Br-cAMP or coexpression of E3KARP. These data show that the inhibition of NHE3 in response to an elevated cAMP level may not involve redistribution of either NHE3 or E3KARP within the resolution of confocal microscopy. However, we cannot rule out a small change in E3KARP or NHE3 distribution undetectable by confocal microscopy, and this requires other quantitative analyses.

Since PDZ domain-containing proteins function as scaffold proteins, we have recently been contemplating a model in which E3KARP and/or NHERF functions as a scaffold. We examined if E3KARP and NHERF might be A kinase anchoring proteins (AKAPs) (25), but found that these regulatory proteins are not (26). Recently, NHERF/EBP50 was shown to bind to ezrin and establish the ezrin-radixin-moesin family proteins (19, 20). Since ezrin has been shown to directly bind PKA (27), the binding of NHERF to ezrin suggested that the NHERF-ezrin complex may be a linker between NHE3 and PKA (19, 20). E3KARP and NHERF share a significant homology, and therefore, we determined if E3KARP is an ezrin-binding protein. Gel overlay study showed that the N-terminal 302 aa of human ezrin bind to both NHERF and E3KARP with comparable affinity. We found that the interaction with ezrin occurs through the C-terminal portion of E3KARP. Our current study further localized the ezrin-binding motif to the C-terminal 23 aa residues (Fig. 8C). Similarly, a recent report showed that ezrin also binds to the carboxyl-terminal 30 aa of human EBP50 (28). A search for proteins with a similar motif did not identify proteins other than NHERF and its homologs, suggesting that this domain of E3KARP may represent a unique ezrin-binding motif. The mechanistic nature of the PKA-dependent inhibition of NHE3 is yet to be determined. The current interpretation of data indicates that the increase in phosphorylation of NHE3 is essential for this inhibition (4). Concurrently, the presence of a regulatory protein, either E3KARP or NHERF, is also necessary. Since ezrin is an AKAP that binds to the reg-

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3 G. Lamprecht and C.-H. C. Yun, unpublished results.

4 G. Lamprecht, E. J. Weinman, and C.-H. C. Yun, manuscript in preparation.
ulatory subunit of type II PKA (27), the binding of ezrin to NHERF suggested a model in which NHERF may scaffold NHE3 and ezrin, which in turn recruit PKA into the vicinity of NHE3, allowing PKA to phosphorylate NHE3. Whether the phosphorylation of NHE3 by PKA and the presence of the regulatory proteins are dependent processes needs further investigation.

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