The Down Regulated in Adenoma (*dra*) Gene Product Binds to the Second PDZ Domain of the NHE3 Kinase A Regulatory Protein (E3KARP), Potentially Linking Intestinal Cl^-/HCO_3^- Exchange to Na^+/H^+ Exchange†

Georg Lamprecht,*,‡ Andreas Heil,‡ Susannah Baisch,‡ Elena Lin-Wu,‡ C. Chris Yun,§ Hubert Kalbacher,|| Michael Gregor,‡ and Ursula Seidler‡

1st Medical Department and Medical and Natural Sciences Research Center, University of Tuebingen, Otfried-Mueller-Strasse 10, 72076 Tuebingen, Germany, and Department of Medicine, Division of Digestive Diseases, Emory University School of Medicine, Atlanta, Georgia 30322

Received April 2, 2002; Revised Manuscript Received July 28, 2002

ABSTRACT: Intestinal electroneutral NaCl absorption is mediated by parallel operation of Na^+/H^+ and Cl^-/HCO_3^- exchange in the enterocyte apical membrane. The ion transporters involved are Na^+/H^+ exchanger 3 (NHE3) and the down regulated in adenoma (*dra*) gene product. cAMP-mediated inhibition of NHE3 requires the transporter to bind to the second PDZ (PSD95, disk large, ZO1) domain of the adapter protein NHE3 kinase A regulatory protein (E3KARP). Because the C-terminal four amino acids of *dra* are ETKF (glutamate-threonine-lysine-phenylalanine), resembling a PDZ interaction motif, we hypothesized that *dra* may also bind to one of the PDZ domains of E3KARP. In vitro the ETKF motif of *dra* binds to the second PDZ domain of E3KARP, the affinity being comparable to that of the known ligand CFTR. The C-terminal phenylalanine, which is an unconventional residue in PDZ interaction motifs, can only be substituted by the classical residue leucine, but not by other hydrophobic residues (valine, isoleucine). Immunofluorescence colocalizes *dra*, NHE3, and E3KARP in the apical compartment of human proximal colon. We suggest a model in which both NHE3 and *dra* bind to the second PDZ domain of E3KARP and that linking of the transporters occurs through dimerization of E3KARP. In such a model, the first PDZ domain would remain available for instance for signal transduction proteins.

In the intestine, NaCl absorption occurs through parallel Na^+/H^+ and Cl^-/HCO_3^- exchange. NHE3[1] is the Na^+/H^+ exchanger isoform involved in this dual exchange (*1, 2*). Recently the down regulated in adenoma (*dra)* gene product has been shown to be defective in congenital chloride diarrhea (*3*), a disease known to involve intestinal chloride, bicarbonate, and water transport (*4*). The *dra* gene product is an intestinal membrane glycoprotein that facilitates chloride, hydroxyl, and oxalate transport (*5*−*8*), and the most recent data indicate that it functions as a Cl^-/HCO_3^- or Cl^-/OH^- exchanger (*9*). NHE3 and *dra* have also been demonstrated in the distal ileum and in the surface cells of the proximal colon on the mRNA and protein level (*10*−*12*). Taken together, these data make the *dra* gene product the most likely candidate for the Cl^-/HCO_3^- exchanger working in parallel with NHE3 in electroneutral NaCl absorption.

Coupling of Na^+/H^+ and Cl^-/HCO_3^- exchange is thought to be mediated by the intracellular pH or by the pH of a common subapical microdomain (*1*, *2*), but further insight into this mechanism has been precluded by the lack of knowledge of the molecular identity of the intestinal anion exchanger.

A number of membrane proteins have been shown to bind to so-called PDZ domains (see reference *13* for a recent review). PDZ domains are protein modules that in most cases bind to the C-terminal three or four amino acids of interacting proteins. The consensus sequence for this interacting motif is E/D-S/T-X-L/V/F. Interaction of internal (i.e., non-C-terminal) motifs with PDZ domains has also been shown in a few cases, but the amino acids involved are not as well characterized.

NHE3 has recently been shown to interact through an internal motif with the second PDZ domain of two closely related proteins, NHERF (NHE3 regulatory factor) (*14*) and E3KARP (NHE3 kinase A regulatory protein) (*15*), which contain two PDZ domains each. Because these proteins also interact through their C-termini with Ezrin, which in turn has been shown by others to be an A kinase anchoring

† Supported by Deutsche Forschungsgemeinschaft Grant La1066/2-1 and by the Fortune-Project of the University of Tuebingen.

* To whom correspondence should be addressed at the 1st Medical Department, University of Tuebingen, 72076 Tuebingen, Germany. Phone: +49 (0) 7071 2987108, fax: +49 (0) 7071 292095; e-mail: hans-georg.lamprecht@uni-tuebingen.de.

‡ 1st Medical Department, University of Tuebingen.

§ Department of Medicine, Emory University School of Medicine.

|| Medical and Natural Sciences Research Center, University of Tuebingen.

[1] Abbreviations: NHE3, sodium proton exchanger isoform 3; *dra*, down regulated in adenoma; CFTR, cystic fibrosis transmembrane regulator; NHERF, NHE3 regulatory factor; EBP50, Ezrin binding protein of 50 kDa (the human homologue of NHERF); E3KARP, NHE3 kinase A regulatory protein; PDZ, PSD-95/disc large/ZO-1; PBS, phosphate-buffered saline; TBS, TRIS-buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; ECL enhanced chemoluminescence; PAGE, polyacrylamide gel electrophoresis; LB, Luria broth; IPTG isopropyl thiogalactoside; TMB, 3,3′,5,5′-tetramethylbenzidine.

10.1021/bi0259103 CCC: $22.00
Published on Web 09/19/2002

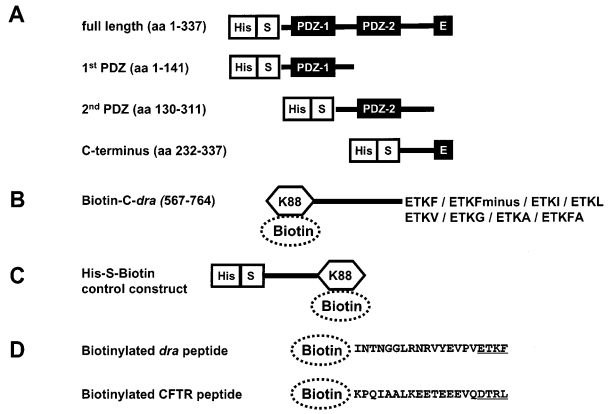

FIGURE 1: Protein constructs and peptides. (A) Full-length E3KARP, its PDZ domains, and its C-terminus were expressed as His-tag/S-tag fusion proteins. (B) The C-terminus of *dra* and mutations of the C-terminal phenylalanine were expressed as fusion proteins with a transcarboxylase tag, which is in *E. coli* biotinylated at lysine-88. (C) The control construct for the interaction ELISA consisted of a fusion protein of the His-tag, the S-tag, and the transcarboxylase-tag. (D) C-terminal peptides of *dra* and CFTR; the PDZ interaction motifs are underlined; control peptides had the underlined amino acids substituted with glycine (not shown).

protein, we proposed a model in which NHERF and E3KARP act as adapter proteins between NHE3 and Ezrin, facilitating the PKA-mediated phosphorylation and subsequent inhibition of NHE3 (*16*, *17*). Because the C-terminal amino acids of *dra* are ETKF (glutamate-threonine-lysine-phenylalanine), which resembles a PDZ domain interaction motif, we hypothesized that *dra* may bind to one of the PDZ domains of E3KARP, which may build a structural link between the functionally coupled Na^+/H^+ and Cl^-/HCO_3^- exchangers.

MATERIALS AND METHODS

Fusion Proteins. Figure 1A shows a cartoon of the fusion protein constructs of E3KARP used in this study. His-tagged constructs of full-length E3KARP, its second PDZ domain, and its C-terminus were used as described (*15*). The first PDZ domain of E3KARP was generated by PCR (*pfu*, Stratagene) using the following primers: sense GAATTCATGGCCGCGCCGGAGC and antisense CTCGAGTCAACTGACATCCTTCTTGCCAGC (*Eco*RI and *Xho*I restriction sites are underlined). The PCR product was cloned into the pCR-II-blunt vector (Invitrogen), sequenced, and then subcloned using the *Eco*RI and *Xho*I restriction sites into pET30 (Novagen). From this vector the constructs are expressed as hexahistidine-tag and S-tag fusion proteins in *E. coli* (BL21). The fusion proteins were affinity-purified under nondenaturing conditions using nickel-nitrilotriacetic acid (NTA) resin as suggested by the manufacturer (Qiagen) and finally eluted in 1 M imidazole, 150 mM NaCl, 10 mM Na_2HPO_4, pH 8. His-tag fusion proteins were quantified using the Lowry procedure with the elution buffer as a blank.

The intracellular C-terminus of *dra* (C-*dra*: amino acids 567−764) and constructs carrying mutations of the C-terminal four amino acids (ETKF minus, ETKI, ETKL, ETKV, ETKG, ETKA, ETKFA) (Figure 1B) were generated by PCR (*pfu*, Stratagene) using the following primers: sense CGAATTCTACGCAAGCGCAAC and antisense TCATTATTAXXXTTTGTTTCAACTGGCACC (X denotes bases coding for the phenylalanine and its mutations) or antisense CATTATTAAACTGGCACCTCATATACCCG (to delete the amino acids ETKF). The PCR product was cloned into pCR-II-blunt TOPO (Invitrogen). The orientation of the insert was determined by restriction analysis; it was sequenced and then subcloned into pinPoint (Promega) using *Bam*HI and *Not*I restriction sites in the pCR-II-blunt vector. The PinPoint vector codes for an N-terminal transcarboxylase tag, which in *E. coli* is biotinylated at lysine-88. To express biotinylated fusion proteins, an overnight culture (pinPoint construct in *E. coli* NM522) was diluted 1:100 in LB plus 50 μM biotin, and after 2.5 h, 0.8 mM IPTG was added. After another 4.5 h, bacteria were spun down and sonicated in 100 mM NaCl, 100 mM Na_2HPO_4, pH 7.4, in a Branson sonifier. The material was finally spun at 20000*g* for 20 min and the supernatant saved.

To obtain a standard curve for the quantitative interaction ELISA (see below) and to estimate the concentration of the biotin-C-*dra* fusion protein (see above and Figure 1B), the transcarboxylase tag from the pinPoint system was cloned into pET30. This facilitated expression and purification (under nondenaturing conditions) of significant amounts of a His-S-biotin fusion protein (Figure 1C), which was quantified using the Lowry protein assay.

Peptides. Peptides comprising the C-terminal 20 amino acids of *dra* (INTNGGLRNRVYEVPVETKF) and CFTR (KPQIAALKEETEEEVQDTRL) and mutated peptides (INTNGGLRNRVYEVPVGGGG and KPQIAALKEETEEEVQGGGG) were synthesized and HPLC-purified at the peptide core facility of the Eberhard-Karls-University Tübingen. All peptides were biotin-labeled at their N-terminus (Figure 1D).

In Vitro Pull Down Assay. His-tag/S-tag constructs of E3KARP (about 4 μg) were diluted in siliconized tubes in 1 mL of interaction buffer (200 mM NaCl, 100 mM NaH_2PO_4, pH 7.5) to decrease the imidazole to less than 15 mM. Then 1 μL (20 μL of 5% suspension supplied by the manufacturer) of magnetic NTA-Ni agarose beads (Qiagen) was added. After 1 h, the beads were separated using a magnet, and the supernatant was removed. The beads were blocked for 10 min using 2% BSA in interaction buffer. Then 500 μL of the cleared bacterial lysate of biotin-C-*dra* or biotin-C-*dra*-ETKFminus and 500 μL of 4% BSA (in interaction buffer) were added, and the suspension was incubated for 4 h. The beads were washed 4 times with interaction buffer using the magnetic separator. Finally the bound material was eluted in 50 μL of Laemmli sample buffer and separated on PAGE. After transfer, the nitrocellulose membranes were blocked with 3% BSA/TBS, and biotinylated proteins were detected using HRP-labeled streptavidin and an ECL-detection system. Essentially the same approach was taken to bind the biotinylated C-terminal peptides of *dra* and CFTR to magnetic streptavidin beads (Promega). These beads were then incubated with E3KARP (expressed from the pET30 vector) which was subsequently detected using an anti-S-tag antibody (Novagen).

Quantitative Interaction ELISA. The quantitative interaction ELISA was performed as a modification of (*18*). His-tag fusion proteins (about 5 pmol) were bound to nickel-coated ELISA plates (Qiagen) overnight. The plates were then washed with 200 μL of PBS/0.1% Tween and blocked with 200 μL of 3% BSA/PBS. Biotinylated peptides (at

various concentrations) or biotin-tag fusion proteins in 100 μL (at about 1 μM; based on comparison of the specific bands with known amounts of the His-S-biotin fusion protein on streptavidin-HRP developed immunoblots) were added for 4 h. After washing and blocking, the plates were incubated for 1 h with 1:250 HRP-streptavidin (Kirkegaard & Perry), washed 6 times with PBS/0.1% Tween, and developed using TMB (Kirkegaard & Perry). To correct for nonlinearity in the upper range of the ELISA, a standard curve was obtained by binding increasing amounts of the His-S-biotin fusion protein (see above) directly to the plate.

Immunofluorescence. Biopsies from proximal colon were prepared as described previously (*12*). An anti-*dra* antibody was raised in chicken (Biogenes GmbH, Berlin, Germany) exactly as previously described for the immunization of rabbits with a *dra* antigen (*12*). The sections were incubated simultaneously overnight at 4 °C with the anti-*dra* antibody (1:50 in PBS plus 1% BSA) and either an anti-E3KARP antiserum (1:125 in PBS plus 1% BSA) raised in rabbit (*15*) or an anti-NHE3 antibody also raised in rabbit (1:500 in PBS plus 1% BSA) (alphaDiagnostics, San Antiona, TX). After washing with PBS, 0.3% Trition X-100, and 0.1% BSA, the sections were incubated simultaneously with Alexa546 coupled anti-rabbit (1:200) and Alexa488 coupled anti-chicken (1:100) secondary antibodies. The samples were washed again and mounted in FluoSave Reagent (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) plus 2.5% diazabicyclo[2.2.2]octane. Sections were examined on a Zeiss Axiovert 135 fluorescence microscope equipped with a PCO Sensicam CCD camera. Antibody dilutions and microscope settings were chosen to exclude significant cross-talk between the two fluorescence channels.

RESULTS

The C-Terminus of dra (ETKF) Is a PDZ Interaction Motif. The interaction of the *dra* C-terminus with E3KARP and its domains was tested in vitro. As noted by others (*19*), initial studies indicated that additional amino acids N- and C-terminal to a PDZ domain are required to facilitate binding of interacting motifs (data not shown). These findings resulted in the design of the constructs of E3KARP shown in Figure 1A.

We immobilized full-length E3KARP, its PDZ domains, and its C-terminus on magnetic agarose beads and tested whether these constructs would precipitate a biotinylated construct of the C-terminus of *dra* from a bacterial lysate (Figure 2). Full-length E3KARP as well as both PDZ domains but not the C-terminus precipitated the wild-type construct of the C-terminus of *dra*. On the other hand, the ETKFminus construct was not precipitated, indicating that the interaction is dependent on the very C-terminal sequence ETKF (glutamate-threonine-lysine-phenylalanine). The differences in the signal obtained from the C-*dra* and the C-*dra*-ETKFminus construct are not a result of unequal biotinylation because equal amounts of the bacterial lysates contained comparable amounts of biotinylated construct (lanes 1 and 2 in Figure 2). Theoretically, the presence of nonbiotinylated C-*dra*-ETKFminus construct competing with the biotinylated construct could explain the results. This potential artifact was ruled out by comparing the streptavidin signal, which detects only biotinylated constructs, with the signal obtained using

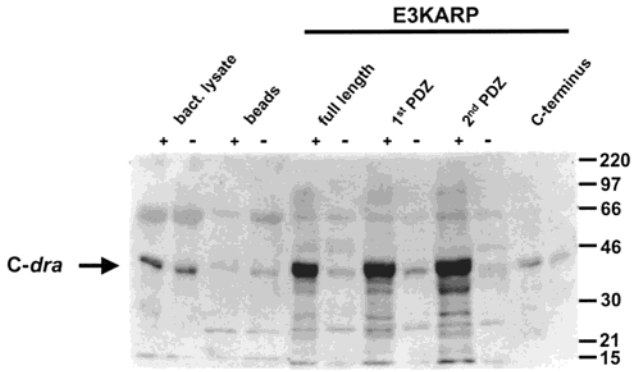

FIGURE 2: The ETKF sequence of *dra* is a PDZ interaction motif. His-tagged fusion proteins of full-length E3KARP, its first and second PDZ domain, and its C-terminus were immobilized on magnetic nickel agarose beads and incubated with bacterial lysates containing a biotinylated fusion protein of the C-terminus of *dra* (C-*dra;* +) and a mutated fusion protein lacking the last four amino acids (C-*dra*-ETKFminus; −). Biotinylated proteins bound to the beads were detected using streptavidin-HRP. Representative blot of 4 experiments.

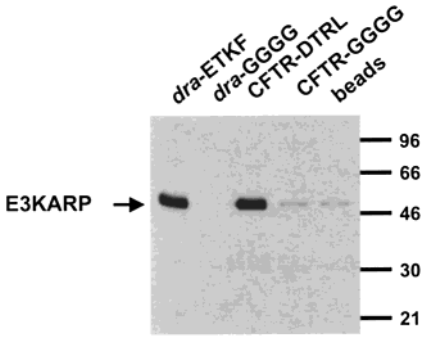

FIGURE 3: *dra* and CFTR bind to E3KARP. 20 amino acid long peptides of *dra* and CFTR, which were biotinylated at their N-termini, were bound to magnetic streptavidin beads and incubated with purified His-S-tagged E3KARP. Bound E3KARP was detected using an anti-S-tag antibody. Representative blot of 3 experiments.

an anti-*dra*-antibody (*12*), which detects the constructs independent of biotinylation. Whether we used the anti-*dra* antibody or streptavidin for detection, the signals from the C-*dra* and the C-*dra*-ETKFminus construct were the same, indicating that competition of a nonbiotinylated construct did not play a role (data not shown).

The reverse experiment tested whether biotinylated C-terminal peptides of *dra* and CFTR immobilized on magnetic streptavidin beads were able to precipitate a soluble construct of E3KARP (Figure 3). The *dra* peptide precipitated E3KARP. The CFTR peptide was used as a positive control and precipitated E3KARP as well. Peptides having the interaction sequences mutated to glycines did not precipitate E3KARP.

Taken together these data indicate that the C-terminal ETKF sequence of *dra* is a PDZ interaction motif.

Mutational Analysis of the Unconventional Phenylalanine in the ETKF Motif. Most PDZ interaction motifs have the general sequence E/D-S/T-X-L/V with the C-terminal residue being tightly bound in a deep cavity of the PDZ domain (*20*). In particular, interaction studies using a random peptide library suggest that the first PDZ domain of NHERF binds to the consensus sequence T-R-L/F and the second PDZ domain of NHERF binds to the consensus sequence S-S/T-

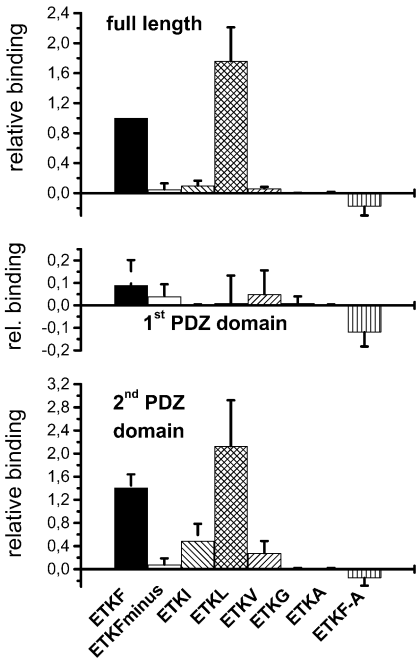

FIGURE 4: Mutational analysis of the PDZ interaction motif of *dra*. The unconventional C-terminal residue phenylalanine of the *dra* PDZ interaction motif was substituted by isoleucine, leucine, valine, glycine, or alanine; an additional alanine was also tested. The interaction with full-length E3KARP and its PDZ domains was tested in an interaction ELISA. The mutations were compared to the wild-type (ETKF) (set as 1) and to the deletion of the entire PDZ interaction motif (ETKFminus). $N = 4$. Note the different scales of the *y*-axis.

W-L (*21*). The PDZ domains of NHERF and E3KARP are highly homologous on the amino acid level, suggesting that they may bind similar motifs, but no experimental studies have addressed what motifs bind to the PDZ domains of E3KARP. Furthermore, CFTR has been shown to bind to the first PDZ domain of NHERF (*22*) but to the second PDZ domain of E3KARP (*23*), indicating that there are subtle differences between the PDZ domains of E3KARP and NHERF with regard to their binding properties. The PDZ interaction motif of *dra* does not match either of the consensus sequences published for the PDZ domains of NHERF and also differs from the C-terminal sequence of CFTR. Therefore, we mutated the C-terminal phenylalanine to hydrophobic (leucine, valine, isoleucine) and to neutral amino acids (glycine and alanine), and we also added an additional alanine. In a semiquantitative interaction ELISA, these constructs were compared to the wild-type sequence as well as to the ETKFminus construct in their ability to bind to full-length E3KARP and to its first and second PDZ domain. The biotinylated constructs of C-*dra* (wild type, ETKFminus, ETKI, ETKL, ETKV, ETKG, ETKA, ETKF-A) were used at the same concentration, which was estimated at 1 μM. The S-tag was used to quantify the amount of the PDZ domain construct actually bound to the plate, and minor differences were corrected for in the analysis. Figure 4 shows that wild-type C-*dra* bound with the highest affinity to full-length E3KARP and to its second PDZ domain but only weakly to its first PDZ domain. Substitution of leucine for phenylalanine resulted in better binding than the wild-type *dra* sequence ($p < 0.001$ for the full-length construct and $p < 0.005$ for the second PDZ domain construct, by ANOVA) but did not affect the binding pattern. On the other hand, substitution of phenylalanine with valine or isoleucine reduced binding dramatically ($p < 0.001$ by ANOVA). Substitution of phenylalanine with the neutral amino acids glycine or alanine as well as addition of an alanine completely disrupted the interaction with any of the E3KARP constructs as did deletion of the entire ETKF sequence. These data show that the unconventional C-terminal phenylalanine specifically facilitates binding of *dra* to the second PDZ domain of E3KARP.

Affinity to the PDZ Domains of E3KARP. To obtain a more quantitative estimate of the binding affinity of the *dra* PDZ interaction motif to the two PDZ domains of E3KARP, increasing amounts of *dra* peptide were incubated in the interaction ELISA, and saturation kinetics were calculated (Figure 5). The CFTR peptide served as a positive control. Consistent with the data from the semiquantitative ELISA, the *dra* peptide bound with high affinity only to the second PDZ domain of E3KARP (1866.7 $\pm$ 381.2 nM, $n = 3$); the affinity to the first PDZ domain was much lower (19 890.5 $\pm$ 7404.7 nM, $n = 3$). As expected, the CFTR peptide bound with high affinity to the second PDZ domain of E3KARP (319.9 $\pm$ 36.9 nM, $n = 3$). The CFTR peptide also bound to a construct comprising the first PDZ domain of NHERF with high affinity (347 $\pm$ 67 nM, $n = 3$), but the *dra* peptide did not bind to that construct (data not shown). These data confirm that *dra* binds to the second PDZ domain of E3KARP. They also confirm that CFTR binds to the second PDZ domain of E3KARP but the first PDZ domain of NHERF.

Colocalization of dra, NHE3, and E3KARP. Immunofluorescence was used to test whether *dra*, NHE3, and E3KARP colocalize in the same subcellular compartment in human proximal colon. *dra* (green label) and NHE3 (red label) immunoreactivity are restricted to the apical plasma membrane (Figure 6A). Superposition of the fluorescence pictures and the phase contrast picture shows that NHE3 and *dra* colocalize in the apical membrane (indicated in yellow). Figure 6B shows colocalization of *dra* (green label) and E3KARP (red label). Specific staining of the apical membrane of surface cells and some upper crypts can be distinguished (arrowheads and arrow in Figure 6B). When the anti-E3KARP antiserum was preincubated with the antigen originally used for immunization (*15*), the membrane staining was almost completely abolished, indicating that staining of subepithelial and intracellular structures by the anti-E3KARP antiserum is nonspecific (data not shown). Again, superposition of the fluorescence pictures and the phase contrast picture demonstrates colocalization of *dra* and E3KARP in the apical membrane of colonic surface cells and upper crypts (indicated in yellow). Taken together, these data indicate that *dra*, NHE3, and E3KARP colocalize in the apical membrane of colonic surface and upper crypt cells.

DISCUSSION

dra Has a PDZ Interaction Motif. NHE3 and *dra* are functionally coupled in the process of electroneutral NaCl absorption in the intestine, and in this process NHE3 binds to the adapter proteins E3KARP and NHERF through an interaction with their second PDZ domains. We studied whether the C-terminal four amino acids of *dra* (ETKF),

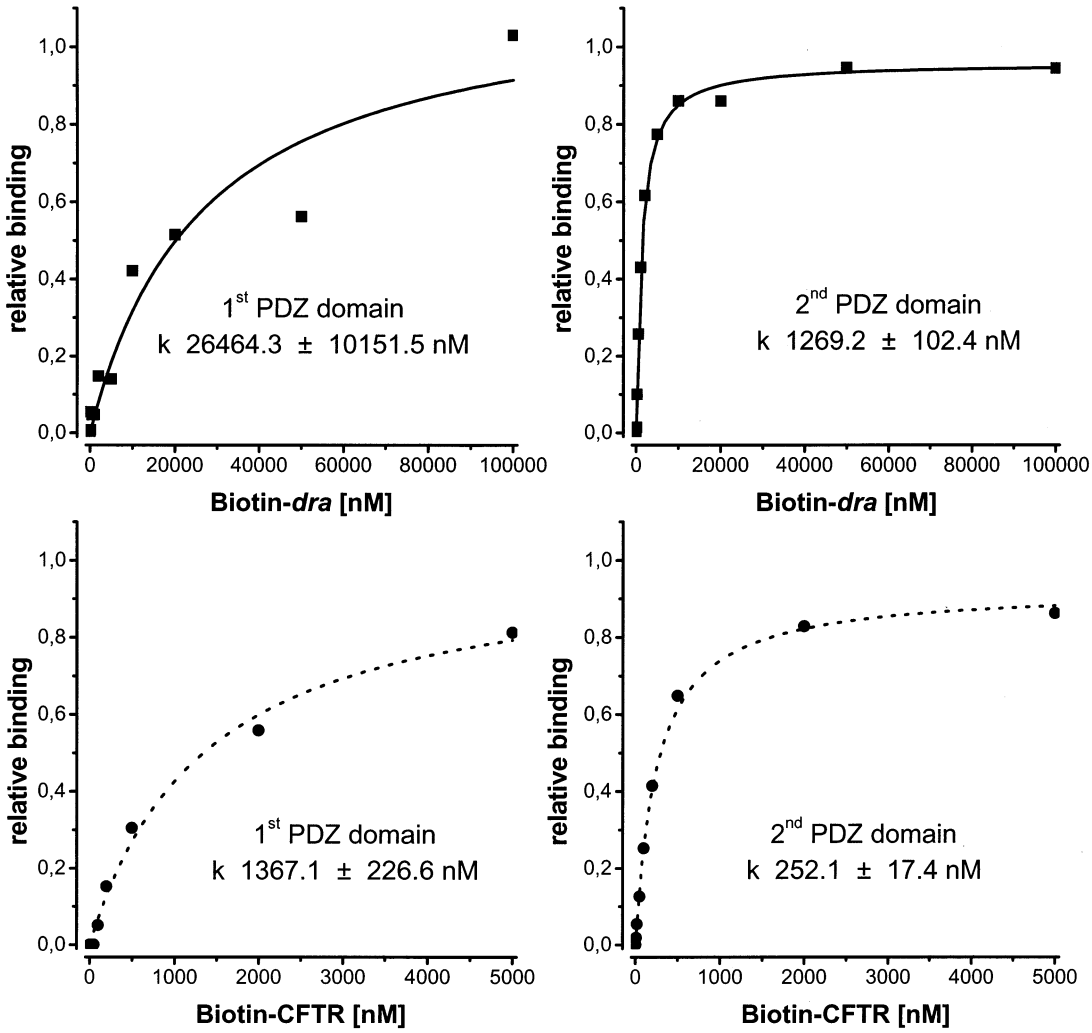

FIGURE 5: Affinity of *dra* and CFTR to the PDZ domains of E3KARP. Increasing concentrations of 20 amino acid long peptides of *dra* and CFTR were used in the interaction ELISA, and saturation kinetics were calculated; maximal binding was set as 1. Peptides with the last four amino acids mutated to glycines did not bind at all (data not shown). Representative of 3 experiments.

which resemble a PDZ interaction motif, might also facilitate interaction with one of the PDZ domains of E3KARP. In the interaction assay using magnetic beads and comparably large amounts of both the PDZ domain constructs and the *dra* constructs (Figure 2), *dra* appeared to interact through its ETKF sequence with both PDZ domains of E3KARP but not with the C-terminal tail of the adapter protein. In the interaction ELISA (Figure 4), which uses substantially less protein, it became apparent that *dra* bound only to the second PDZ domain of E3KARP and that this binding was very specific, because the C-terminal phenylalanine (F) could only be substituted by a leucine (L). Taken together, these data strongly indicate that the C-terminal ETKF sequence of *dra* comprises a PDZ interaction motif that binds specifically to the second PDZ domain of E3KARP. While studying the interaction of CFTR with NHERF, others have noted that in an overlay experiment a CFTR peptide bound to both PDZ domains of NHERF and that only a more sophisticated assay (surface plasmon resonance) was able to differentiate between high-affinity binding to the first PDZ domain and low-affinity binding to the second PDZ domain (*22*). Therefore, we used the ELISA to quantify the interaction. The biotinylated constructs of the entire C-terminus of *dra* (C-*dra*, Figure 1B) could not be used to determine absolute binding affinities, because they could not be purified from the bacterial lysates. Instead we used biotinylated peptides (Figure 1D) in the quantitative ELISA (Figure 5). The *dra* peptide bound with the highest affinity to the second PDZ domain of E3KARP (1889 nM). Our positive control, the CFTR peptide, bound with a k_D of 318 and 389 nM to the first PDZ domain of NHERF (data not shown) and the second PDZ domain of E3KARP. Binding of CFTR to the second PDZ domain of E3KARP was not unexpected since it has been observed before (*23*). Using similar CFTR peptides and surface plasmon resonance as a technique to study interaction, Short et al. have determined a k_D of 40 nM (*22*) and Wang et al. a k_D of 48 nM (*21*) for the first PDZ domain of NHERF.

Several factors have to be considered when interpreting the affinity constant of *dra* to the second PDZ domain of E3KARP: (1) Compared to the affinity constants for CFTR determined by surface plasmon resonance (*21*, *22*), our ELISA appears to be less sensitive by a factor of 8. (2) It has been noted by others that affinities determined using peptides are sometimes underestimated (*24*, *25*). (3) E3KARP and NHERF have recently been shown to dimerize (*26*−*28*), and dimerization of NHERF appears to be regulated through binding of CFTR to the first PDZ domain and maybe also through phosphorylation of serine-289 (*27*). Such dimerization may affect binding to the PDZ domains. We

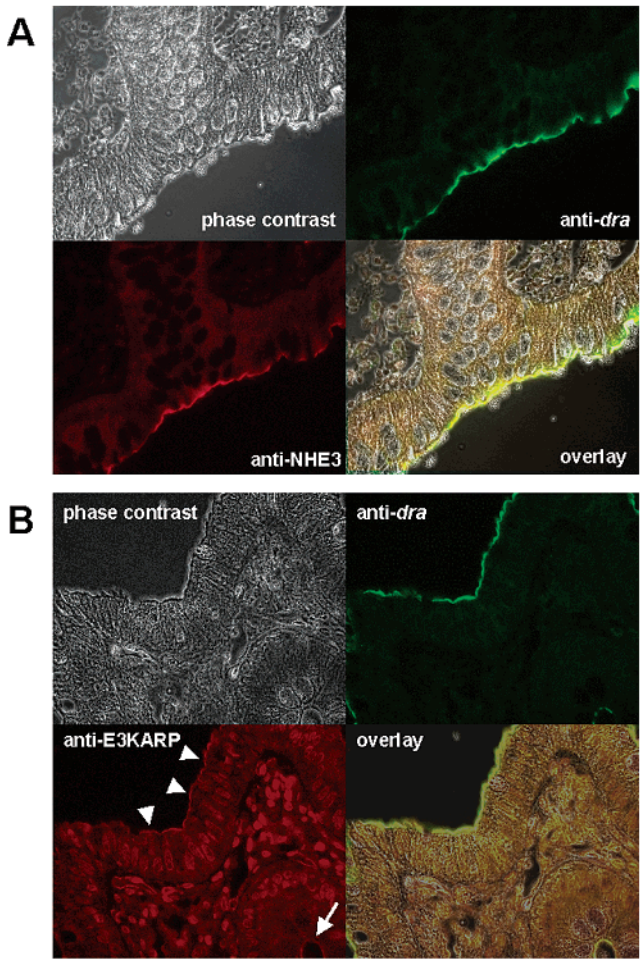

FIGURE 6: Localization of *dra*, NHE3, and E3KARP in human proximal colon. Dual labeling immunofluorescence using anti-*dra* (raised in chicken, detected with Alexa488-labeled anti-chicken) and anti-NHE3 or anti-E3KARP (both raised in rabbit, detected with Alexa546-labeled anti-rabbit) was performed on biopsies from proximal colon. (A) *dra* (green label) and NHE3 (red label) are only detected in the apical compartment of surface cells, where they colocalize (indicated by the yellow color on overlaid pictures). (B) *dra* (green label) and E3KARP (red label) are also colocalized in the apical membrane of surface cells and upper crypts (indicated by the yellow color on overlaid pictures). Staining of subepithelial structures by the anti-E3KARP antiserum is nonspecific (data not shown).

do not know whether binding of our constructs to the ELISA wells facilitates or affects dimerization. Thus, the binding affinity to the individual PDZ domains of E3KARP may be underestimated. Taking these factors into account, our data suggest that the affinity of *dra* for the second PDZ domain of E3KARP can be physiologically relevant.

Potential Physiological Significance. One of the reasons why we believed that *dra* might bind to E3KARP was that such an interaction would put *dra* in close proximity with NHE3, which in turn might enhance or facilitate the known functional coupling (*1*, *2*) of the two transporters. This hypothesis was based on the known localization of *dra*, NHE3, and E3KARP in ileum and proximal colon (*5*, *10*−*12*, *29*−*32*). Nevertheless, none of these studies has directly examined the colocalization of these proteins. Figure 6 shows that *dra* colocalizes with NHE3 and with E3KARP in the apical compartment of proximal colonic surface cells, where *dra* is most highly expressed (*5*, *12*). The colocalization of the three proteins in native tissue strongly suggests that their interaction is physiologically important.

In some cases, functionally related or coupled membrane proteins have been shown to bind to two distinct PDZ domains of the same interacting protein, thereby forming a direct link, while in many other cases two different transporters bind to the same PDZ domain of one interacting protein and the linking may occur more indirectly (reviewed in *13*). NHE3 has been shown to bind to the second PDZ domain of E3KARP (*15*) and NHERF (*14*). Our data indicate that *dra* also binds to the second PDZ domain of E3KARP. Nevertheless, a structural link between NHE3 and *dra* may be formed by E3KARP, because this adapter protein has been shown to dimerize (*27*, *28*), creating two second PDZ domains for interaction. In such a complex, NHE3 and *dra* (each binding to one second PDZ domain) as well as Ezrin [bound to the C-terminus of E3KARP (*15*, *23*)] and protein kinase A [bound to Ezrin (*33*)] would be in close proximity. Upon stimulation by cAMP, protein kinase A phosphorylates and inhibits NHE3 (*16*, *17*). The transport activity of *dra* is probably inhibited indirectly through changes of the pH of the intracellular microdomain surrounding the complex (*1*, *2*), because *dra* does not have a PKA consensus site.

In such a model, the first PDZ domain of E3KARP remains available for other interacting proteins, a number of which have been described (*34*). But many of these are not present in or near the apical plasma membrane of epithelial cells. Recently, EPI64 (EBP50-PDZ interactor of 64 kDa), a protein predicted to have a role in apical membrane fusion, has been shown to bind to the first PDZ domain of NHERF/EBP50 and E3KARP (*34*). It is tempting to speculate that EPI64 and other signal transduction proteins (*35*) may target their effect on ion transporters (e.g., *dra* and NHE3) through binding to a common adapter protein like NHERF/EBP50 or E3KARP.

ACKNOWLEDGMENT

This report includes experimental work performed by Andreas Heil and Elena Lin-Wu in fulfillment of the requirements for their doctoral theses.

REFERENCES

1. Knickelbein, R., Aronson, P. S., Atherton, W., and Dobbins, J. W. (1983) Sodium and chloride transport across rabbit ileal brush border. I. Evidence for Na−H exchange. *Am. J. Physiol. 245*, G504−510.
2. Knickelbein, R., Aronson, P. S., Schron, C. M., Seifter, J., and Dobbins, J. W. (1985) Sodium and chloride transport across rabbit ileal brush border. II. Evidence for Cl−HCO_3 exchange and mechanism of coupling. *Am. J. Physiol. 249*, G236−245.
3. Hoglund, P., Haila, S., Socha, J., Tomaszewski, L., Saarialho-Kere, U., Karjalainen-Lindsberg, M. L., Airola, K., Holmberg, C., de la Chapelle, A., and Kere (1996) Mutations of the Down-regulated in adenoma (DRA) gene cause congenital chloride diarrhoea. *Nat. Genet. 14*, 316−319.
4. Kere, J., Lohi, H., and Hoglund, P. (1999) Genetic Disorders of Membrane Transport. III. Congenital chloride diarrhea. *Am. J. Physiol. 276*, G7−G13.
5. Byeon, M. K., Westerman, M. A., Maroulakou, I. G., Henderson, K. W., Suster, S., Zhang, X. K., Papas, T. S., Vesely, J., Willingham, M. C., Green, J. E., and Schweinfest, C. W. (1996) The down-regulated in adenoma (DRA) gene encodes an intestine-specific membrane glycoprotein. *Oncogene 12*, 387−396.
6. Byeon, M. K., Frankel, A., Papas, T. S., Henderson, K. W., and Schweinfest, C. W. (1998) Human DRA functions as a sulfate transporter in Sf9 insect cells. *Protein Expression Purif. 12*, 67−74.

7. Moseley, R. H., Hoglund, P., Wu, G. D., Silberg, D. G., Haila, S., de la Chapelle, A., Holmberg, C., and Kere, J. (1999) Downregulated in adenoma gene encodes a chloride transporter defective in congenital chloride diarrhea. *Am. J. Physiol. 276*, G185−G192.
8. Silberg, D. G., Wang, W., Moseley, R. H., and Traber, P. G. (1995) The Down regulated in Adenoma (dra) gene encodes an intestine-specific membrane sulfate transport protein. *J. Biol. Chem. 270*, 11897−11902.
9. Melvin, J. E., Park, K., Richardson, L., Schultheis, P. J., and Shull, G. (1999) Mouse Down-regulated in Adenoma (DRA) Is an Intestinal Cl/HCO_3 Exchanger and Is Up-regulated in Colon of Mice Lacking the NHE3 Na^+/H^+ Exchanger. *J. Biol. Chem. 274*, 22855−22861.
10. Dudeja, P. K., Rao, D. D., Syed, I., Joshi, V., Dahdal, R. Y., Gardner, C., Risk, M. C., Schmidt, L., Bavishi, D., Kim, K. E., Harig, J. M., Goldstein, J. L., Layden, T. J., and Ramaswamy, K. (1996) Intestinal distribution of human Na^+/H^+ exchanger isoforms NHE-1, NHE-2, and NHE-3 mRNA. *Am. J. Physiol. 271*, G483−G493.
11. Hoogerwerf, W. A., Tsao, S. C., Devuyst, O., Levine, S. A., Yun, C. H. C., Yip, J. W., Cohen, M. E., Wilson, P. D., Lazenby, A. J., Tse, C. M., and Donowitz, M. (1996) NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. *Am. J. Physiol. 270*, G29−G41.
12. Jacob, P., Rossmann, H., Lamprecht, G., Kretz, A., Neff, C., Lin Wu, E., Gregor, M., Groneberg, D. A., Kere, J., and Seidler, U. (2002) Down-regulated in adenoma mediates apical Cl^-/HCO_3^- exchange in rabbit, rat, and human duodenum. *Gastroenterology 122*, 709−724.
13. Sheng, M., and Sala, C. (2001) PDZ domains and the organization of supramolecular complexes. *Annu. Rev. Neurosci. 24*, 1−29.
14. Weinman, E. J., Steplock, D., Donowitz, M., and Shenolikar, S. (2000) NHERF associations with sodium−hydrogen exchanger isoform 3 (NHE3) and ezrin are essential for cAMP-mediated phosphorylation and inhibition of NHE3. *Biochemistry 39*, 6123−6129.
15. Yun, C. H. C., Lamprecht, G., Foster, D. V., and Sidor, A. (1998) NHE3 Kinase A regulatory protein (E3KARP) binds the epithelial brush border Na/H exchanger, NHE3, and the cytoskeletal protein ezrin. *J. Biol. Chem. 273*, 25856−25863.
16. Lamprecht, G., Weinman, E. J., and Yun, C. H. (1998) The role of NHERF and E3KARP in the cAMP-mediated inhibition of NHE3. *J. Biol. Chem. 273*, 29972−29978.
17. Zizak, M., Lamprecht, G., Steplock, D., Tariq, N., Shenolikar, S., Donowitz, M., Yun, C. H. C., and Weinman, E. J. (1999) cAMP-induced Phosphorylation and Inhibition of Na^+/H^+ Exchanger 3 (NHE3) Are Dependent on the Presence but Not the Phosphorylation of NHE Regulatory Factor. *J. Biol. Chem. 274*, 24753−24758.
18. Kashishian, A., Howard, M., Loh, C., Gallatin, W. M., Hoekstra, M. F., and Lai, Y. (1998) AKAP79 inhibits calcineurin through a site distinct from the immunophilin-binding region. *J. Biol. Chem. 273*, 27412−27419.
19. Raghuram, V., Mak, D. O., and Foskett, J. K. (2001) Regulation of cystic fibrosis transmembrane conductance regulator single-channel gating by bivalent PDZ-domain-mediated interaction. *Proc. Natl. Acad. Sci. U.S.A. 98*, 1300−1305.
20. Karthikeyan, S., Leung, T., and Ladias, J. A. A. (2001) Structural Basis of the Na^+/H^+ Exchanger Regulatory Factor PDZ1 Interaction with the Carboxyl-terminal Region of the Cystic Fibrosis Transmembrane Conductance Regulator. *J. Biol. Chem. 276*, 19683−19686.
21. Wang, S., Raab, R. W., Schatz, P. J., Guggino, W. B., and Li, M. (1998) Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). *FEBS Lett. 427*, 103−108.
22. Short, D. B., Trotter, K. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R. C., Stutts, M. J., and Milgram, S. L. (1998) An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J. Biol. Chem. 273*, 19797−19801.
23. Sun, F., Hug, M. J., Lewarchik, C. M., Yun, C.-H. C., Bradbury, N. A., and Frizzell, R. A. (2000) E3KARP Mediates the Association of Ezrin and Protein Kinase A with the Cystic Fibrosis Transmembrane Conductance Regulator in Airway Cells. *J. Biol. Chem. 275*, 29539−29546.
24. Brenman, J. E., Christopherson, K. S., Craven, S. E., McGee, A. W., and Bredt, D. S. (1996) Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. *J. Neurosci. 16*, 7407−7415.
25. Yanagisawa, J., Takahashi, M., Kanki, H., Yano-Yanagisawa, H., Tazunoki, T., Sawa, E., Nishitoba, T., Kamishohara, M., Kobayashi, E., Kataoka, S., and Sato, T. (1997) The Molecular Interaction of Fas and FAP-1. A Tripeptide Blocker of Human Fas Interaction with FAP-1 Promotes Fas-Induced Apoptosis. *J. Biol. Chem. 272*, 8539−8545.
26. Shenolikar, S., Minkoff, C. M., Steplock, D. A., Evangelista, C., Liu, M., and Weinman, E. J. (2001) N-terminal PDZ domain is required for NHERF dimerization. *FEBS Lett. 489*, 233−236.
27. Lau, A. G., and Hall, R. A. (2001) Oligomerization of NHERF-1 and NHERF-2 PDZ domains: differential regulation by association with receptor carboxyl-termini and by phosphorylation. *Biochemistry 40*, 8572−8580.
28. Fouassier, L., Yun, C. C., Fitz, J. G., and Doctor, R. B. (2000) Evidence for ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) self-association through PDZ−PDZ interactions. *J. Biol. Chem. 275*, 25039−25045.
29. Li, X., Galli, T., Leu, S., Wade, J. B., Weinman, E. J., Leung, G., Cheong, A., Louvard, D., and Donowitz, M. (2001) Na^+-H^+ exchanger 3 (NHE3) is present in lipid rafts in the rabbit ileal brush border: a role for rafts in trafficking and rapid stimulation of NHE3. *J. Physiol. (London) 537*, 537−552.
30. Rajendran, V. M., Black, J., Ardito, T. A., Sangan, P., Alper, S. L., Schweinfest, C., Kashgarian, M., and Binder, H. J. (2000) Regulation of DRA and AE1 in rat colon by dietary Na depletion. *Am. J. Physiol. 279*, G931−G942.
31. Rocha, F., Musch, M. W., Lishanskiy, L., Bookstein, C., Sugi, K., Xie, Y., and Chang, E. B. (2001) IFN-gamma downregulates expression of Na^+/H^+ exchangers NHE2 and NHE3 in rat intestine and human Caco-2/bbe cells. *Am. J. Physiol. 280*, C1224−C1232.
32. Yun, C. H. C., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C. M., Weinman, E. J., and Donowitz, M. (1997) cAMP-mediated inhibition of the epithelial brush border Na^+/H^+ exchanger, NHE3, requires an associated regulatory protein. *Proc. Natl. Acad. Sci. U.S.A. 94*, 3010−3015.
33. Dransfield, D. T., Bradford, A. J., Smith, J., Martin, M., Roy, C., Mangeat, P. H., and Goldenring, J. R. (1997) Ezrin is a cyclic AMP-dependent protein kinase anchoring protein. *EMBO J. 16*, 35−43.
34. Reczek, D., and Bretscher, A. (2001) Identification of EPI64, a TBC/rabGAP domain-containing microvillar protein that binds to the first PDZ domain of EBP50 and E3KARP. *J. Cell Biol. 153*, 191−206.
35. Tang, Y., Tang, J., Chen, Z., Trost, C., Flockerzi, V., Li, M., Ramesh, V., and Zhu, M. X. (2000) Association of mammalian Trp4 and phospholipase C isozymes with a PDZ domain-containing protein, NHERF. *J. Biol. Chem. 275*, 37559−37564.

BI0259103