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The CFTR Associated Protein CAP70 Interacts with the Apical Cl^-/HCO_3^- Exchanger DRA in Rabbit Small Intestinal Mucosa

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ABSTRACT: DRA (down regulated in adenoma) is an intestinal anion exchanger, acting in parallel with NHE3 to facilitate ileal and colonic NaCl absorption. Furthermore it is involved in small intestinal bicarbonate secretion. Because DRA has a PDZ interaction motif, which may influence its properties, we searched for DRA-interacting PDZ adapter proteins in the small intestine. Using an overlay assay with the recombinant DRA C-terminus as a ligand, a 70 kDa protein was labeled, which was restricted to the brush border membrane in rabbit duodenal and ileal mucosa and was not detected in the colon. Destruction of the C-terminal PDZ interaction motif abolished this band, suggesting a specific protein−protein interaction. The 70 kDa protein was identified as CAP70 (CFTR associated protein of 70 kDa) by an anti-CAP70 antibody and by two in vitro binding assays after cloning CAP70 from rabbit duodenum and ileum. The interaction was recapitulated in HEK cells transfected with DRA and PDZK1, the human orthologue of CAP70. Corresponding to the overlay assay, no CAP70 mRNA or protein was detected in the colon. In vitro protein−protein interaction studies revealed specific binding of DRA to the 2nd and 3rd PDZ domain, while CFTR is known to interact with PDZ1, PDZ3, and PDZ4. The composition of macromolecular complexes assembled by CAP70 in the distal small bowel is unknown. Its restricted expression shows that it cannot be involved in NaCl absorption in the proximal colon. We suggest that CAP70 mediates regulatory functions specific to the small intestine.

The small intestinal mucosa, in humans predominantly the duodenal mucosa, secretes HCO_3^-. One part of this HCO_3^- secretion is mediated by CFTR-dependent HCO_3^- channels or, more likely, by CFTR[1] itself (cystic fibrosis transmembrane conductance regulator) (*1*, *2*). CFTR is known to be a Cl^- channel and is mutated in patients suffering from cystic fibrosis (*3*). The other part of the HCO_3^- secretory process is electroneutral and is mediated by a Cl^-/HCO_3^- exchanger (*4*, *5*). Its molecular identity has been under debate for a long time, but recent data suggest that two members of the SLC26 anion transporter gene family, DRA (down regulated in adenoma; also called SLC26A3 (*6*)) and PAT1 (putative anion transporter; also called SLC26A6 (*7*)) are involved.

In 1993 DRA was cloned by subtractive hybridization, and was shown to be down regulated in adenomas compared to normal colonic mucosa (*8*). While the significance of DRA for the adenoma−carcinoma sequence of colonic neoplasia is not yet clear (*9*), Hoglund et al. have shown that mutations in the SLC26A3 gene cause congenital chloride diarrhea (CLD), a rare genetic disorder, which is attributable to dysfunction of Cl^-/HCO_3^- exchange in the colon (*10*). DRA, which is highly expressed in rabbit and human small and large intestine, transports Cl^-, HCO_3^-, OH^-, and SO_4^{2-} (*6*, *11*−*14*). Expression studies and isotope flux studies have provided evidence that in the distal ileum and proximal colon the coupled activity of DRA and NHE3 (Na^+/H^+ exchanger

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[1] Abbreviations: CAP70, CFTR associated protein of 70 kDa.; DRA, down regulated in adenoma; PDZK1, PDZ domain containing protein K1, human orthologue of CAP70; CFTR, cystic fibrosis transmembrane conductance regulator; NHE3, Na/H exchanger isoform 3; BBM, brush border membrane; BLM, basolateral membrane; SLC, solute carrier; PAT1, putative anion transporter 1; NHERF, NHE3 regulatory factor; E3KARP, NHE3 kinase A regulatory protein; RACE, rapid amplification of cDNA ends; ETKF, glutamate−threonine−lysine−phenylalanine; SGLT1, Na-dependent glucose transporter 1; f.l., full length; AE2, anion exchanger isoform 2; PDZ, PSD-95/disclarge/ZO-1; STATS, sulfate transporter and anti-sigma antagonist; SH2, Src homology 2; SH3, Src homology 3; URAT1, uric acid transporter 1, NaP_i-IIa, Na phosphate cotransporter type IIa.

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isoform 3) results in NaCl reabsorption (*15*, *16*), while in the duodenum high expression levels of DRA and low expression levels of NHE3 may result in HCO_3^- secretion (*6*).

Patients suffering from cystic fibrosis show impaired small intestinal and pancreatic HCO_3^- secretion (*17*), and the low HCO_3^- secretory rates contribute to malabsorption and obstruction in these organs. Mutations in CFTR often affect CFTR-activated HCO_3^- transport more severely than Cl^- transport (*17*). On the basis of this somewhat indirect evidence that CFTR influences Cl^-/HCO_3^- exchange (at least in part mediated by DRA), several concepts have been developed postulating that such coupling occurs on either a functional (*18*), a transcriptional (*19*), or a structural (*20*) level. Ko et al. recently reported C-terminal PDZ motif dependent coprecipitation of DRA and CFTR and suggest an influence of the SLC26 transporters, including DRA, on CFTR gating (*20*, *21*).

CFTR, DRA, and PAT1 all have PDZ interaction motifs, which facilitate binding to intracellular PDZ adapter proteins (*22*−*24*). PDZ adapter proteins possess one or more PDZ domains and often contain additional protein−protein interaction domains such as coiled-coil domains, ERM domains, or SH2 or SH3 domains (*25*). CAP70 (the human orthologue is called PDZK1), for example, has four PDZ domains (*26*), three of which facilitate binding of CFTR. CAP70 has been shown to cluster two CFTR molecules resulting in the potentiation of channel activity (*22*). CAP70 has been identified using various approaches and has thus also been called CLAMP (*27*), diphor-1 (*28*), and NaP_i Cap1 (*29*).

In general there is currently no unifying concept for the role of the different PDZ adapter proteins in gastrointestinal ion transport. One hypothesis is that different PDZ adapter proteins facilitate the organization of different multiprotein complexes each with a specific function. Because DRA is believed to fulfill different functions in the proximal small bowel compared to the ileum and proximal colon (*6*, *10*), we have searched for interacting PDZ domain proteins in these segments of the gastrointestinal tract. Here we describe the interaction of CAP70 with DRA in the proximal and distal small intestine but not in the colon.

EXPERIMENTAL PROCEDURES

Cloning of the 5′ and 3′ Ends of Rabbit DRA and of the Complete Coding Sequence of Rabbit CAP70. Total cellular RNA was isolated from rabbit duodenal, ileal, and colonic mucosa, and first strand cDNA was synthesized using either SuperScript II reverse transcriptase (Invitrogen GmbH, Karlsruhe Germany) or the SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, CA) anchoring a known sequence to the 5′ and 3′ ends of transcribed cDNAs.

Gene specific primers were chosen from the published partial coding sequence of rabbit DRA (Table 1) (*30*). The 5′ and 3′ cDNA regions were amplified using one gene specific primer and a universal primer, complementary to the anchor sequence, for each PCR reaction (SMART RACE cDNA Amplification Kit, BD Biosciences Clontech, Palo Alto, CA). The obtained amplimers were cloned in pCR2.1 TOPO (Invitrogen GmbH, Karlsruhe Germany) and sequenced. The nucleotide sequence of rabbit DRA, deposited in GenBank with the accession number AF314819, was updated.

For cloning of full length rabbit CAP70 degenerated primers were deduced from published sequence information for human and mouse CAP70 (Table 1). After cloning and sequencing of the PCR product as described above, gene specific primers were chosen form the obtained sequence (Table 1) and 5′ and 3′ RACE PCR and cloning of the cDNA fragments were carried out as detailed above. The nucleotide sequence of full length rabbit CAP70 has been deposited in the GenBank database under GenBank accession number AY204473.

PDZK1, the human orthologue of CAP70, was cloned from an ileal biopsy using primers (Table 1) deduced from the published sequence (GenBank accession number AF012281). The PCR product was cloned into pCR-II-blunt (Invitrogen GmbH, Karlsruhe Germany), sequence verified, and subcloned into pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA) using *Bam*HI and *Xba*I (*Xba*I originated form the cloning vector pCR-II-blunt).

Preparation of Rabbit Duodenal, Ileal and Colonic Brush Border (BBM) and Basolateral (BLM) Membranes. Rabbit BBM and BLM vesicles were prepared by a combination of differential- and density-gradient centrifugation as described in ref *30*. The BBM fractions were enriched ∼23-fold in alkaline phosphatase (Na^+/K^+ ATPase: about ∼1.9-fold), and the BLM fractions were enriched ∼15-fold in Na^+/K^+ ATPase (alkaline phosphatase: ∼0.9-fold) (*6*). The purity of the membrane fractions and the integrity of the proteins were confirmed by Western blot analysis using an anti-SGLT1 and an anti-Na^+/K^+-ATPase antibody. (*6*).

Fusion Proteins. His-tagged (pET30, Novagen, Madison, WI) and GST-tagged (pGEX6P2, Amersham Biosciences, Freiburg, Germany) constructs of the 198 C-terminal amino acids of human and rabbit DRA (C-DRA ETKF+) and a deletion mutant lacking the last 4 amino acids (C-DRA ETKF−) were generated as described in ref *23*. Figure 1 shows the amino acid sequence of rabbit CAP70 and the PDZ domain sequences as determined by PFSCAN (Husar 5.0, DKFZ, Heidelberg, Germany). Full length CAP70 (f.l. CAP70) and its PDZ domains were amplified using primers and PCR conditions (polymerase: PFU, Stratagene, La Jolla, CA) detailed in Table 1. The PCR products were cloned into the pCR-II-blunt vector (Invitrogen GmbH, Karlsruhe, Germany), sequenced, and then subcloned into pET30 (Novagen, Madison, WI) using the *Bam*HI and *Xho*I restriction sites.

Both His-tag/S-tag fusion proteins (pET30) and GST-tag fusion proteins (pGEX6P) were expressed in *Escherichia coli* (BL21). The His-tag fusion proteins were affinity-purified under nondenaturing conditions using nickel-nitrilotriacetic acid (NTA) resin as suggested by the manufacturer (Qiagen, Hilden, Germany), and the GST-tag fusion proteins were purified using Glutathion sepharose, again as suggested by the manufacturer (Amersham Biosciences, Freiburg, Germany). Fusion proteins were quantified using the Lowry or the Bradford procedure.

Western and Far Western Analysis. Cell lysates (60 μg of protein per lane in lysis buffer: 1 mM EDTA, 0.1% SDS, 1% Nonidet P 40, 1 mM PMSF in PBS, pH 7.3), purified BBMvs, BLMvs (60 μg of protein per lane), and fusion proteins (20 pmol of protein per lane) were resolved on SDS−PAGE. Prior to loading, cell lysates and purified membrane protein were heated to 42 °C for 1 min and

Table 1: Primers Used for RT-PCR, RACE-PCR, Construct Generation, and Expression Analysis[a]

primer name	primer sequence	amplimer	annealing	derived from
	Primers for Cloning the 5′ and 3′ Ends of Rabbit DRA			
D_GSP1.for	GTGGCGTTTGAACTCCTCACCATCG	≥1207 bp (3′)	65−48 °C, TD, 45 s	AF314819
Universal Primer	Short (SMART RACE cDNA Amplification Kit)			Clontech
D_GSP2.rev	CACGGGAAGCTTGGCTTTGAAGC	≥1048 bp (5′)	70−58 °C, TD, 45 s	AF314819
Universal Primer	Short (SMART RACE cDNA Amplification Kit)			Clontech
	Primers for Cloning of the Rabbit C-DRA Constructs ETKF+/ETKF−			
C-DRA.for	**CATATGGATCCATG**GCTATTGGCTTCAGCCCC		64−56 °C, TD, 30 s	AF314819
ETKF+.rev	**CTCGAGTCATTATTA**GAACTTCGTTTCAATTGGGACC	650 bp		
ETKF−.rev	**CTCGAGTCATTATTA**AATTGGGACCTGATATTCCC	638 bp		
	Primers for Cloning Full Length Rabbit CAP70			
C_het_1.for	ATGRCCTCCACCTTCAACCCC	1039 bp	60−46 °C, TD, 45 s	AF0122811/
C_het_2.rev	CATTRGGCAGTTCTTGACTTTG			AF220100
C_het_1.for	ATGRCCTCCACCTTCAACCCC	1411 bp	60−46 °C, TD, 45 s	AF0122811/
C_het_3.rev	CRATRGGGATTTTCTTAGCTTGG			AF220100
C_GSP1.for	GGTGGACAGAATCCAAAGCAGTGGG	≥1023 bp (3′)	65−48 °C, TD, 45 s	AY204473
Universal Primer	Short (SMART RACE cDNA Amplification Kit)			Clontech
C_hom.for	ATTAGTCTGTGGAAAGAAGGCC	626 bp (3′)	60−46 °C, TD, 45 s	AY204473
C_het_4.rev	CCACARCATTGYTACAAAAGCAC			AF0122811/ AF220100
C_GSP2.rev	CCCTTCCTCATCTCCACAATGCGG	≥779 bp (5′)	65−48 °C, TD, 45 s	AY204473
Universal Primer	Short (SMART RACE cDNA Amplification Kit)			Clontech
	Primers for the Generation of CAP70 Constructs			
f.l.CAP70.for	**GGATCC**ATGGCCTCCACCTTCAAC	1557 bp	60−46°, TD, 240 s	AY204473
f.l.CAP70.rev	**CTCGAGTTA**CAGCTCGGTATCCTCAGAATTGG			
PDZ1.for	**GGATCC**ATGACCTCCACCTTCAACCCCC	336 bp	51°, 45 s	AY204473
PDZ1.rev	**CTCGAGTCA**CGCCTCCTGCCGACTCTGACC			
PDZ2.for	**GGATCC**TTGAAAGAGTTGGGTCAGAGTCGG	372 bp	51°, 45 s	AY204473
PDZ2.rev	**CTCGAGTCA**TATCTTCTGCTCGCTGTGCCG			
PDZ3.for	**AAAGGATCC**GAGAAACGGCACAGC	432 bp	55°, 120 s	AY204473
PDZ3.rev	**CTCGAGTCA**CGGAGGAACAGGGGTAGGAGC			
PDZ4.for	**CATATGGATCCATG**GCGGACAGCATGTATAGACTGG	485 bp	56 °C, 60 s	AY204473
PDZ4.rev	**CTCGAGTCATTATTA**AGCCATGGAGGAAACAATAGGG			
PDZ2/3.for	**CATATGGATCCATG**AAAGAGTTGGGTCAGAGTCGG	790 bp	58 °C, 60 s	AY204473
PDZ2/3.rev	**CTCGAGTCATTATTA**GGTAGGAGCTGCAACCTCC			
PDZ3/4.for	**CATATGGATCCATG**CGTATCGTGTTCCTTCTGGTG	755 bp	58 °C, 60 s	AY204473
PDZ3/4.rev	**CTCGAGTCATTATT**ATTCTTAGCTTGGAAATATTCGTAGG			
	Primers for Cloning Full Length Human PDZK1			
PDZK1.for	CTCTT**GGATCC**CCAGAAATGACCTCCACC	1596 bp	60−48 °C, TD, 4 min	AF0122811
PDZK1.rev	**AAGCTT**TTACTTGTTTTCATCACATCTCTG			
	Primers for DRA Expression Analysis			
RabDRA for.	CCACAGACCCTCTAACAAC	498 bp	54 °C, 60 s	AF314819
RabDRA rev.	ACCCTTTCTCCATCTTCCC			

[a] Sequence in bold letters: sequence added to facilitate cloning. Right column: GenBank accession numbers. Clontech: BD Biosciences Clontech, Palo Alto, CA. TD: touch down protocol.

recombinant protein was boiled for 1 min. Proteins were electroblotted to nitrocellulose or PVDF membranes (Amersham Biosciences, Freiburg, Germany) and stained with Ponceau S. Membranes were blocked in 5% nonfat dry milk in TBS Triton (150 mM NaCl, 13 mM Tris, 0.02% Triton X-100, pH 7.5) for 1 h, incubated with the diluted primary antibody (anti-DRA 1:1000 (*6*) or anti-CAP70 1:5000 (*29*)) or an antigen/antibody complex (C-DRA ETKF+/anti-DRA, C-DRA ETKF−/anti-DRA, f.l. CAP70/anti-CAP70, GST-tagged fusion protein/anti-GST [goat anti-GST, Amersham Biosciences, Freiburg, Germany]; see below) overnight at 4 °C, washed in TBS Triton, and incubated with a horseradish peroxidase-(HRP) conjugated goat anti-rabbit IgG antibody (1:5000) or a horseradish peroxidase-(HRP) conjugated donkey anti-goat IgG antibody (1:2000) (both from Jackson ImmunoResearch Lab.; West Grove, PA). Bands were detected by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany). To obtain quantitative information the films were scanned and the bands analyzed by Scion Image Beta 4.0.2 (Scion Corporation, Frederick, MD).

To generate the antigen/antibody complexes for Far Western analysis anti-DRA was diluted 1:1600, anti-CAP70 1:3200. anti-GST 1:20000 in block solution and 1.35 μg/mL recombinant protein (His-tagged C-DRA ETKF+, His-tagged C-DRA ETKF−, His-tagged f.l. CAP70, GST-tagged C-DRA ETKF+, GST-tagged C-DRA ETKF−) was added. The mixture was incubated at 37 °C for 1 h while being shaken continuously.

Coimmunoprecipitation. Subconfluent untransfected HEK cells (HEK/−) and HEK cells stably expressing a N-terminal EGFP-tagged human DRA construct (EGFP-DRA-ETKF+) or an EGFP-tagged DRA construct lacking the C-terminal PDZ interaction motif (EGFP-DRA-ETKF−) were transiently transfected in a 25 cm^2 flask with 10 μg of pEGFP/PDZK1 using 20 μL of Lipofectamine 2000 according to the recommendations of the manufacturer (Invitrogen). Untransfected HEK cells (HEK/−) also served as negative control. Cells were grown for 24 h and were then lysed in 1000 μL of cell lysis buffer (100 mM NaCl, 50 mM NaF, 20 mM TRIS, pH 7.5 plus 1% Triton X-100 and Complete

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  1  MASTFNPREC KLSKEEGQNY GFFLRIEKDT EGHLVRVIEK GSPAEKAGLQ
                              PDZ1
 51  DGDRVLRING VFVDKEEHMQ VVDLVRKSGN AVTLLVLDGN SYEKAVKKQV

101  DLKELGQSRQ EADLRDENVA PVMNGGVETW TQPRLCYLEK QGNSYGFSLK
                              PDZ2
151  TVQGKKGVYM TDIIPQSVAM KAGVLADDHL IEVNGENVED ASHEEVVEKV

201  KKSGNRIVFL LVDKETEKRH SEQKIEFRRE AASLKLLPHQ PRIVEMKKGS
                              PDZ3
251  SGYGFYLKAG PEQRGQIIKD IDSGSPAEAA GLKNNDLVIA VNGKSVEALD

301  HDGVVELIKK GGDQTSLLVV DKEADSMYRL AHFSPFLYYQ SQELPNGSVT

351  EVAAPTPVPP EVSSPDPTEE VEDHKPKLCR LDKGENGYGF HLNAIRGLPG
                              PDZ4
401  SFVKEVQKGS PADLAGLEDE DIIIEVNGVN VLDEPYEKVV DRIQSSGDNV

451  TLLVCGKKAY EYFQAKKIPI VSSMALPLAI PADSQGMLAE LEYNLHEAKE

501  RAHSTASNSS SNSEDTEL*
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FIGURE 1: Amino acid sequence of rabbit CAP70, PDZ domains, and protein constructs. Rabbit CAP70 was cloned by RT-PCR using heterologous primers and a RACE protocol from rabbit ileal mucosa. The nucleotide sequence has been deposited in the GenBank database under GenBank accession number AY204473. PDZ domain consensus sequences as determined by PFSCAN (Husar 5.0, DKFZ, Heidelberg, Germany) are given in bold letters. Full length rabbit CAP70, each of its four PDZ domains, and the double constructs PDZ2/3 and 3/4 were expressed as His-tag fusion proteins (see Table 1).

protease inhibitor cocktail without EDTA [Roche]). The lysate was spun 5 min at 10.000 g, and the supernatant was incubated with 10 μL of rabbit anti-DRA antiserum for 3 h. Then 25 μL of washed protein-A-sepharose beads (Amersham) was added and the incubation continued for another 60 min. The beads were washed 3 times in cell lysis buffer, and the material bound to the beads was eluted by boiling in 50 μL of Laemmli sample buffer for 3 min. Proteins were separated on 8.5% PAGE and blotted onto nitrocellulose. Both EGFP-DRA-ETKF+ (or EGFP-DRA-ETKF−) and EGFP-PDZK1 were detected using a monoclonal anti-EGFP antibody (JL-8, 1:500, Clontech) and an anti-mouse secondary antibody (1:5000, Jackson Immunochemicals). Actin was detected by an anti-actin antibody (catalog number A-2066; Sigma, Taufkirchen, Germany) and an anti-rabbit secondary antibody (1:5000).

Quantitative Interaction ELISA. The quantitative interaction ELISA was performed as described previously (*23*). Briefly His- and S-tagged fusion proteins (expressed from pET30) of full length CAP70 and its PDZ domain constructs were immobilized on nickel-coated ELISA plates. The S-tag was used to quantify the amount of His-tagged CAP70 construct actually bound to the plate, and minor differences were corrected for. A biotin fusion protein of the 198 C-terminal amino acids of DRA (Bio-C-DRA) was incubated with the CAP70 constructs and was detected using Streptavidin-HRP.

RESULTS

Evidence for a DRA-Binding Protein. In order to identify DRA interacting proteins, 60 μg of highly purified basolateral (BLM) and apical (BBM) membrane protein from rabbit duodenal and ileal mucosa were resolved by SDS−PAGE. The blot was overlaid with a recombinant protein comprising the DRA C-terminus, which had been preincubated with an anti-DRA antibody. Under these conditions a strong 70 kDa band was detected by the C-DRA/anti-C-DRA complex in the brush border but not in the basolateral membrane fractions both of duodenum and of ileum (Figure 2A, left panel). In the absence of the C-DRA protein the anti-DRA antibody expectedly localized DRA in the brush border membrane fraction of duodenum and ileum (108 to 116 kDa, Figure 2A, middle panel) (*6*). Because rabbit protein was used in this experiment, anti-rabbit IgG secondary antibody was applied without primary antibody and disclosed several nonspecific bands (Figure 2A, right panel).

This observation and the fact that the C-terminal four amino acids (ETKF) of DRA are known to form a PDZ domain interaction motif (*23*) raised the question of whether a specific protein−protein interaction occurs between this motif and the putative 70 kDa DRA-binding protein. Duodenal BBM protein (Figure 2B) was run on two parallel blots. Incubation of the first blot with the C-DRA/anti-C-DRA complex (including the last 4 amino acids: C-DRA ETKF+) again revealed the 70 kDa band (Figure 2B, left panel). But incubation of the other blot with a deletion mutant of the C-DRA protein lacking the last four amino acids (C-DRA ETKF−) did not result in the 70 kDa band (Figure 2B, right panel). As observed for the wild type protein the deletion mutant of C-DRA was able to completely block the specific DRA bands at 108 and 116 kDa (compare Figure 2A left and middle panels), demonstrating that C-DRA ETKF− is recognized by the anti-DRA antibody. Also independent experiments show that the anti-DRA antiserum recognizes C-DRA ETKF+ and C-DRA ETKF− with equal affinity (data not shown).

These data suggest that a 70 kDa, PDZ domain(s) containing protein is present in small intestinal BBM, which is able to bind to the C-terminus of the DRA protein.

The Molecular Identity of the Observed DRA-Binding Protein. Because DRA is an anion exchanger, which in the distal small bowel and in the proximal colon functions in parallel with the Na^+/H^+ exchanger NHE3 to facilitate electroneutral NaCl absorption (*9*), two NHE3-binding proteins were considered as candidate proteins for the DRA-interacting band: NHERF (*31*) and E3KARP (*32*). Both adapter proteins contain PDZ domains, and for E3KARP binding to DRA has already been shown (*23*). Comparison

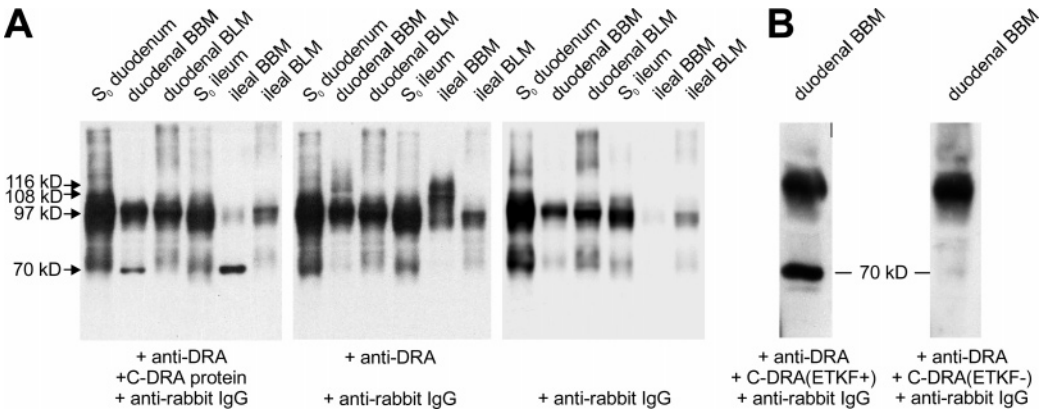

FIGURE 2: Evidence for a DRA-binding protein. A: Total mucosal homogenate (S0), as well as basolateral (BLM), and apical (BBM) membrane protein from rabbit duodenal and ileal mucosa were resolved by SDS− PAGE. Western blot experiments using an anti-DRA antibody (middle panel) localized DRA in the apical membrane fractions (97, 108, and 116 kDa). Preincubation of the anti-DRA antibody with the recombinant C-DRA protein (left panel) blocked the DRA signal completely. Simultaneously a strong 70 kDa band appeared in duodenum and ileum, which was restricted to the brush border membrane fraction. Incubation of the blot with secondary antibody only identified nonspecific bands (right panel). Representative of eight experiments. B: Two parallel blots with duodenal BBM protein were prepared. Incubation with the C-DRA protein (including the last 4 amino acids: ETKF+) bound to the anti-DRA antibody again revealed the 70 kDa band (left panel). Deletion of the C-terminal PDZ interaction motif (ETKF−) abolished binding to the 70 kDa band (right panel) suggesting that the specific protein−protein interaction between DRA and the DRA-binding protein is mediated by one or several PDZ domains. Representative of four experiments.

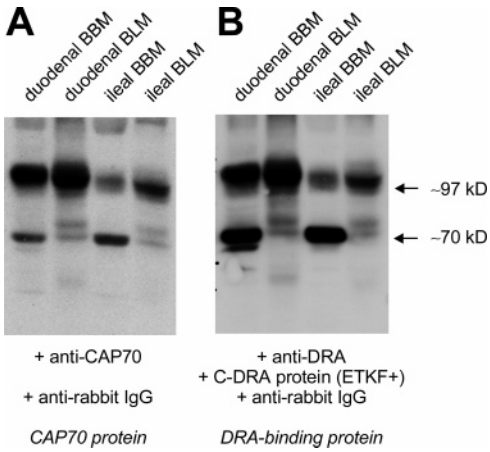

FIGURE 3: The DRA-binding protein, detected by Far Western experiments, is identical with CAP70. CAP70 (A) and the DRA-binding protein (B) both have a molecular weight of 70 kDa, are restricted to the brush border membrane fraction of duodenum and ileum, display higher expression in the ileal than in the duodenal epithelium, and show a double band in the duodenum and only one band in the ileum. Representative of three experiments.

of Western blots (using rabbit duodenal and ileal membrane preparations and an anti-NHERF and an anti-E3KARP antibody) with Far Western experiments (as described above) reveals binding of C-DRA to both adapter proteins, but under these conditions to a lesser extent (data not shown). Therefore we searched the database for a PDZ domain(s) containing protein with a calculated molecular weight of about 70 kDa and present in the apical compartment of enterocytes. Because of the proposed coupling of DRA and CFTR, CFTR-binding adapter proteins were also searched for. CAP70 is a CFTR anchoring protein of 70 kDa, containing 4 PDZ domains (*22*), and therefore CAP70 seemed to be a good candidate for the DRA-binding protein. Figure 3A shows duodenal and ileal brush border as well as basolateral membrane protein, incubated with an anti-CAP70 antibody. Compared to the DRA-binding protein (Figure 3B) CAP70 displays the same molecular weight and the same band pattern (double band at about 70 kDa in duodenum, single band at about 70 kDa in ileum). Taken together these data strongly suggest that CAP70 is the 70 kDa protein interacting with DRA.

PDZK1 Interacts with Human DRA through Its PDZ Interaction Motif in Vivo. To test the interaction of DRA and PDZK1, the human orthologue of CAP70, in vivo, HEK293 cells were stably transfected with human DRA carrying a N-terminal EGFP-tag (EGFP-DRA-ETKF+) or a mutant lacking the PDZ interaction motif (EGFP-DRA-ETKF−). PDZK1 was transiently transfected and was also N-terminally tagged with EGFP. Native HEK293 cells and HEK293 cells only transfected with EGFP-PDZK1 served as controls. We chose the human DRA and PDZK1 construct in this case to preserve a consistent human system by transfection in HEK293 cells and to show that interaction occurs in rabbit and human. Lysates from all four cell lines were immunoprecipitated using an anti-DRA antibody, and the precipitate was checked for the presence of EGFP-DRA and EGFP−PDZK1 using an anti-EGFP antibody.

Figure 4 shows that EGFP-DRA+ and EGFP-DRA− were equally expressed in the two clonal cell lines and were effectively precipitated by the anti-DRA antibody. EGFP-DRA was detected both in the lysates and in the immunoprecipitates as two bands of 145 kDa and about 180 kDa. The lower band corresponds to the band previously described by us in a nonclonal HEK293 cell line (*12*). The higher band may be related to the high expression of EGFP-DRA in these clonal cell lines. The molecular weight of EGFP-PDZK1 is 86 kDa and almost exactly corresponds to the calculated weight of PDZK1 (57.1 kDa) plus the EGFP-tag (26.9 kDa).

EGFP-PDZK1 was coprecipitated with EGFP-DRA-ETKF+. In the precipitate from the HEK/EGFP-DRA-ETKF− cells only minimal EGFP−PDZK1 signal was detected, which was not different from the signal detected in HEK cell lacking DRA, indicating that this is unspecific background and further indicating that the interaction of DRA and PDZK1 occurred through the PDZ interaction motif. Specificity of the bands is shown by the absence of any EGFP signal in the untransfected HEK cells. The amount of

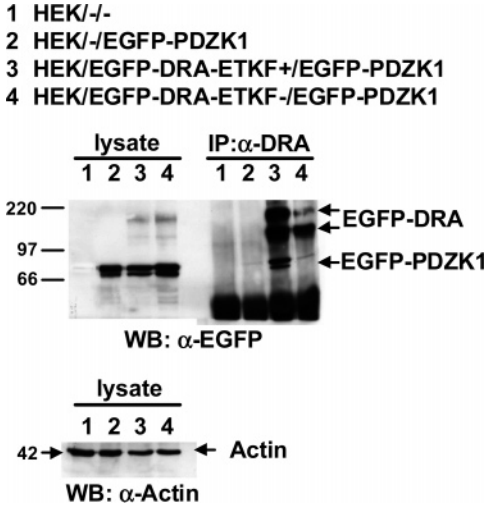

FIGURE 4: DRA interacts in vivo with PDZK1 (the human orthologue of CAP70) through its PDZ interaction motif ETKF. Untransfected HEK cells (HEK/−) and HEK cells stably transfected with EGFP-DRA-ETKF+ or EGFP-DRA-ETKF− were additionally transiently transfected with EGFP-PDZK1. DRA was immunoprecipitated, and the precipitate was checked for the presence of EGFP-PDZK1. EGFP-PDZK1 was only coimmunoprecipitated from EGFP-DRA-ETKF+ expressing cells but not from EGFP-DRA-ETKF− expressing cells or cells lacking any DRA. Note that EGFP-PDZK1 is expressed as two bands similar to the expression of CAP70 in the duodenum. Equal loading of the lanes is demonstrated by anti-actin antibody. Representative of six experiments.

PDZK1 coprecipitated with DRA varied from experiment to experiment such that no firm conclusion with regard to the stoichiometry could be drawn, but there was never more EGFP-PDZK1 than EGFP-DRA detected.

Due to technical problems coimmunoprecipitation of DRA and CAP70 using membranes from rabbit tissue turned out to be impossible: The antibody used to precipitate DRA and the antibody to detect native, i.e., untagged, CAP70 are both raised in rabbits. Thus the rabbit IgG introduced to precipitate DRA led to background problems and did not allow CAP70 to be detected.

CAP70 mRNA and Protein Expression Is Low in Colonic Mucosa Compared to Small Intestine. DRA expression in rabbit colonic mucosa is at least as high as in rabbit duodenal mucosa (*6*). PDZK1 (the human orthologue of CAP70), however, has been shown not to be expressed in human colon (*22*, *26*). Therefore the expression of CAP70 in the colon was compared to that in the duodenum using RT-PCR, Western blot, and Far Western analysis. In contrast to the situation in the duodenum and ileum, neither CAP70 mRNA (Figure 5A) nor protein expression (Figure 5B, lower panel) or the described overlay band (Figure 5B, upper panel) was observed in rabbit colon.

Complete Coding Sequence of Rabbit DRA and Rabbit CAP70. In order to test the interaction of DRA and CAP70 in vitro first the full length nucleotide sequences of rabbit DRA and rabbit CAP70 (Figure 1) were determined (GenBank accession numbers: DRA, updated version of AF314819; CAP70, AY204473). Both sequences showed high homology to published sequence information of the corresponding proteins of other species, e.g., human DRA/rabbit DRA 84.3% (human DRA/mouse DRA 84.2%) and human CAP70/rabbit CAP70 89.4% (human CAP70/mouse

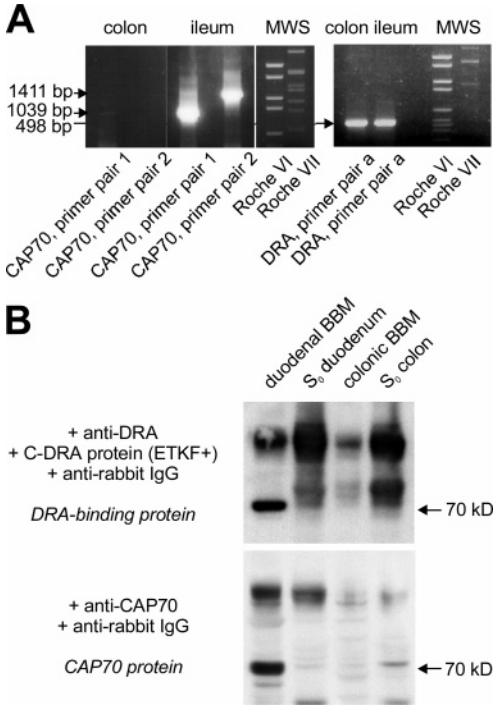

FIGURE 5: Compared to small intestine CAP70 mRNA and protein expression is low level in colonic mucosa. A: Two different primer pairs almost failed to amplify a CAP70 PCR product from rabbit proximal colonic total RNA, while the same primers amplified CAP70 cDNA fragments from rabbit ileal total RNA isolations without any problem (left panel, 40 cycles; primer pair1, C_het_1.for/C_het_2.rev; primer pair 2, C_het_1.for/C_het_3.rev; for sequence information see Table 1). From the ileal as well as from the colonic RNA preparations DRA PCR products were obtained easily (right panel, 35 cycles; primer pair a, rabDRA.for/rabDRA.rev; for sequence information see Table 1). The identity of the amplimers was confirmed by cloning and sequencing. PCR experiments were repeated for both CAP70 and DRA, using at least two further primer pairs. B: In contrast to the ileum, Far Westerns loaded with colonic BBM protein and incubated with the C-DRA/anti-DRA antigen/antibody complex failed to detect an overlay band in the colonic BBM membrane fraction (upper panel). Consistent with this finding is the absence of a CAP70 protein band in the Western blot shown in the lower panel. Representative of two experiments.

CAP70 83.1%). The C-terminal four amino acids of the DRA protein (ETKF), which form the PDZ domain interaction motif (*23*), are conserved in all analyzed species (human, mouse, rat).

The different expression pattern of CAP70 and DRA in rabbit duodenum and ileum on one hand and rabbit colon on the other hand raised the question, whether different DRA variants are expressed in small and large intestine. 5′ and 3′ RACE PCR products for DRA were obtained from rabbit duodenal, ileal, and colonic cDNA. Sequencing of the PCR products showed sequence identity of several 5′ and 3′ DRA clones from duodenal, ileal, and colonic mucosa.

The C-Terminus of DRA Binds to Full Length CAP70 and the Second and Third PDZ Domains of CAP70 in Far Western Interaction Studies Using Recombinant Proteins. To confirm that the C-terminus of DRA in fact binds to CAP70 and to find out which PDZ domains are responsible for this interaction, full length CAP70, each of its 4 PDZ domains, and the double constructs PDZ2/3 and PDZ3/4 were cloned into pET30 (Figure 1, Table 1), expressed as His-tagged fusion proteins in BL21, were affinity purified, separated by SDS−PAGE, blotted, and incubated with the C-DRA/

antibody complex (Figure 6A,B). The blots on the left were overlaid with the wild type C-DRA construct (ETKF+), and the blots on the right were overlaid with the deletion mutant of C-DRA (lacking the last 4 amino acids, ETKF−). Of note the positive and negative blot each originated from the same gel, that had been blotted to the same membrane, had been cut and processed in parallel, and which were finally exposed to the same film. Protein−protein interaction of CAP70 with the DRA C-terminus could easily be demonstrated for full length CAP70 and for the second PDZ domain (Figure 6A). No signal was detected for the first, third, and fourth PDZ domains using single PDZ domain constructs (Figure 6A). Quantitative analysis of the blot shown in Figure 6B (optical density of the bands on the positive blot minus the optical density of the bands on the negative control blot), however, revealed a slightly stronger binding of the double construct PDZ2/3 compared to PDZ2. Furthermore there was also some binding to PDZ3/4. From these two observations we conclude that PDZ3 may also be involved in the interaction of CAP70 with the C-terminus of DRA.

The highly specific binding of C-DRA to full length CAP70 was confirmed when C-DRA (ETKF+) and C-DRA (ETKF−) proteins were separated by SDS−PAGE, blotted, and overlaid with a CAP70/anti-CAP70 antigen/antibody complex. Binding was only detected for the ETKF+ construct, but not for the ETKF− construct (Figure 6C).

Binding of DRA to CAP70 and Its PDZ Domains by Quantitative Interaction ELISA. To complement the Far Western data by another more quantitative method and to obtain more information about the role of PDZ3 we tested the interaction of DRA with CAP70 in an interaction ELISA. Figure 7 shows that DRA bound with the highest affinity to the full length construct and with comparable affinity to the second and the third PDZ domain. There was no significant binding to the fourth PDZ domain and only little binding to the first PDZ domain. As in the overlay assay the double constructs did not reconstitute the binding affinity to the full length protein. CFTR has been shown to bind to the first, third, and fourth PDZ domains of CAP70. Our constructs, which differ slightly from those used by Wang et al. (*22*), proved to bind CFTR as well indicating that all PDZ domain constructs were functional (data not shown). Also there was no significant binding of the C-DRA ETKF− construct to any of the PDZ domain constructs.

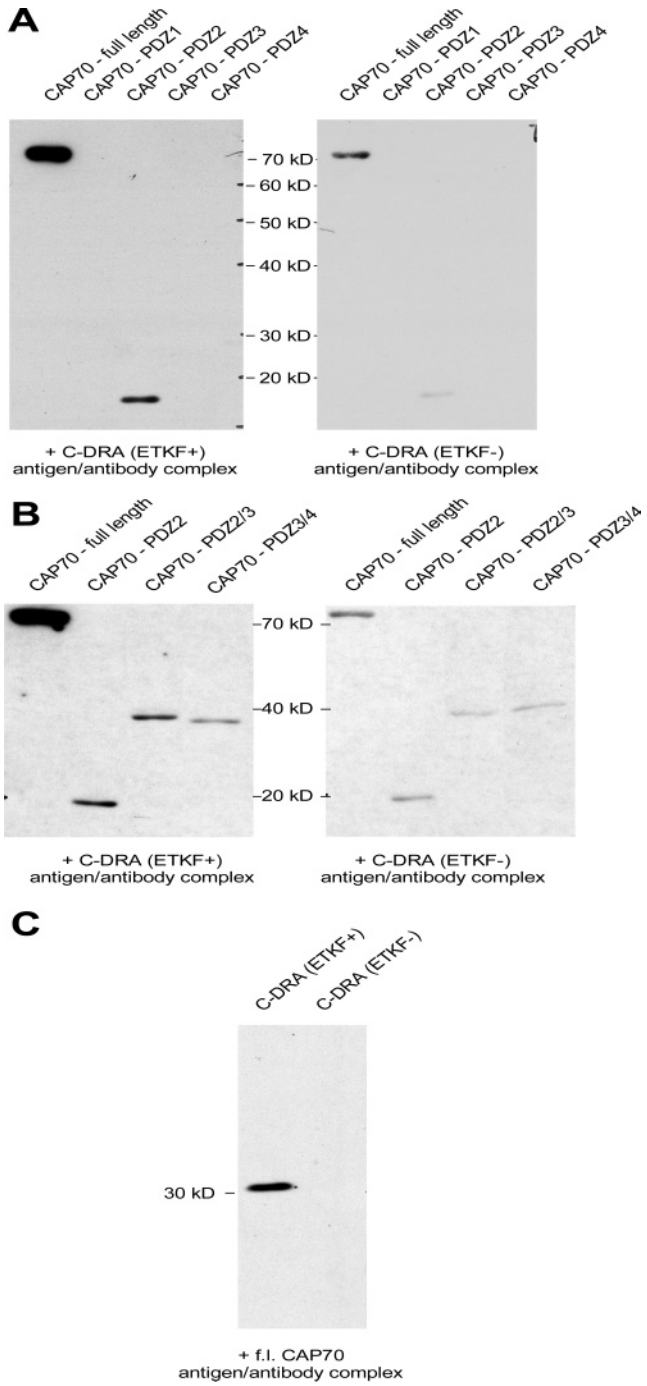

FIGURE 6: The C-terminus of DRA binds to full length CAP70 and the secnd and third PDZ domains of CAP70 in Far Western interaction studies using recombinant protein. A: Full length CAP70 and each of its 4 PDZ domains were separated by SDS−PAGE, blotted, and incubated with the C-DRA/antibody complex. The blot shown in the right panel was overlaid with the wild-type C-DRA construct (ETKF+), and the blot shown in the left panel was overlaid with the deletion mutant of C-DRA (lacking the last 4 amino acids, ETKF−). Representative of five experiments. B: Full length CAP70, PDZ2, and the double constructs PDZ2/3 and PDZ3/4 were separated by SDS−PAGE, blotted, and incubated with the C-DRA/antibody complex. The blot shown in the right panel was overlaid with the wild-type C-DRA construct (ETKF+), and the blot shown in the left panel was overlaid with the deletion mutant of C-DRA (lacking the last 4 amino acids, ETKF−). Representative of two experiments. C: C-DRA (ETKF+) and C-DRA (ETKF−) proteins were separated by SDS−PAGE, blotted, and incubated with the CAP70/anti-CAP70 antibody complex. *N* = 1.

DISCUSSION

DRA is a recently discovered intestine specific apical anion exchanger (*10*, *13*, *14*). As such it is involved both in electroneutral NaCl absorption in the distal small bowel and in the proximal colon (*10*) as well as in electroneutral bicarbonate secretion in the duodenum (*6*). DRA has a C-terminal class I PDZ interaction motif (*23*). Such PDZ interaction motifs have been implicated in the apical targeting, the apical recycling, the clustering, and the specific regulation of several other transmembrane proteins (for review see refs *33*−*35*). The current study was aimed to identify and characterize DRA-interacting protein(s) in the small intestine.

Initially a 70 kDa DRA-binding protein was identified by the blot overlay (Far Western) of small intestinal brush border membrane protein with the DRA C-terminus and an anti-

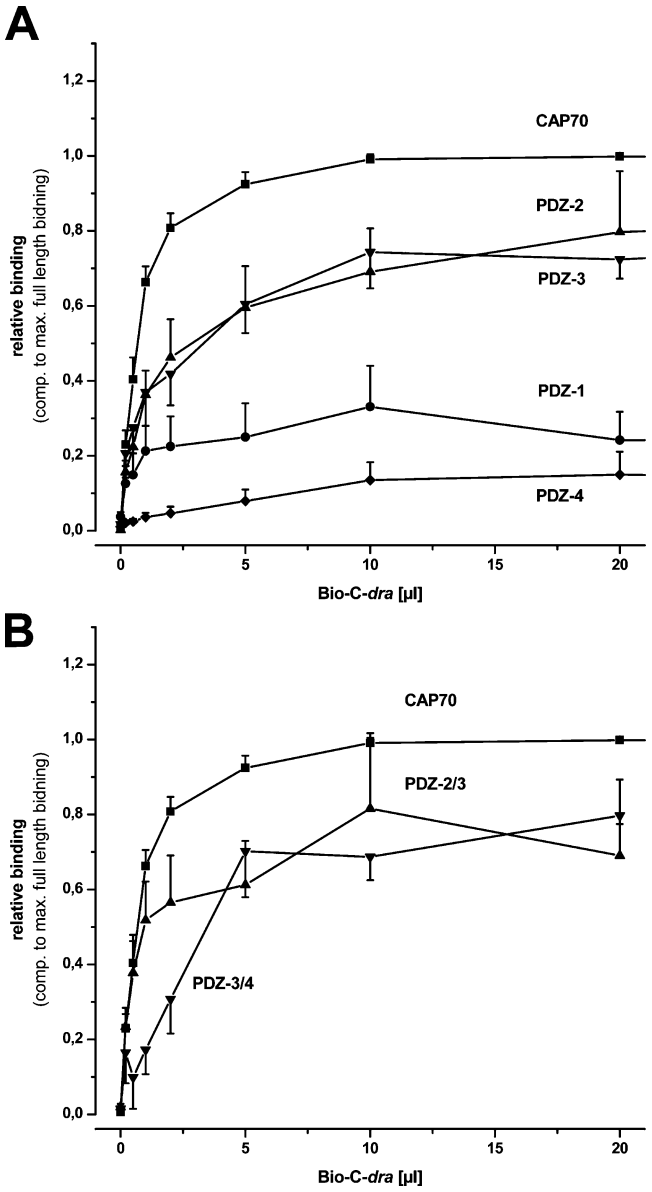

FIGURE 7: The C-terminus of DRA binds to full length CAP70 and the secnd and the third PDZ domain of CAP70 in an interaction ELISA. Full length CAP70 and its individual PDZ domains were expressed as His-tagged, S-tagged fusion proteins and bound to nickel-coated ELISA plates. Differences in the amount of fusion protein bound to the plate were corrected for using the S-tag and a S-protein-HRP conjugate. Increasing concentrations of Biotin-C-DRA were used to test the affinity to full length CAP70 and the different PDZ constructs. A: Full length CAP70 and the single domain PDZ constructs. B: Full length CAP70 and the PDZ2/3 and the PDZ3/4 construct. $N = 4-14$, error bars are SEM.

DRA antibody. Several lines of evidence indicate that this 70 kDa DRA-interacting protein is CAP70: (1) The DRA-binding protein and CAP70 show exactly the same molecular weight, and (2) they display the same band pattern (two bands in the duodenum, one band in the ileum). (3) The DRA-binding protein and CAP70 are both present in the small intestine but not in the large intestine. (4) CAP70 mRNA is highly expressed in the duodenal and ileal epithelium, and the CAP70 cDNA was cloned from both tissues. (5) The interaction was reproduced using recombinant proteins in two different in vitro assays (Far Western and ELISA) and by coimmunoprecipitation of DRA and PDZK1 in HEK293 cells. (6) Binding occurs in rabbit and human model systems.

The DRA-binding protein observed in these experiments was not identical with the NHE3-binding adapter proteins NHERF and E3KARP. The interaction of the DRA C-terminus with the latter has already been demonstrated (*23*), and Far Western experiments using small intestinal membrane protein suggest that the DRA C-terminus binds to both adapter proteins, but in the overlay assay to a lesser extent (data not shown).

A DRA deletion mutant lacking the C-terminal four amino acids (ETKF) (Figure 2) does not label the 70 kDa band and does not facilitate protein−protein interaction with CAP70 in the in vitro assays. Because these four amino acids represent a PDZ domain binding motif (*23*), which is conserved in all known species, the interaction of DRA with CAP70 occurs through one or several of its PDZ domains. The in vitro binding assays indicate that at least the second and also the third PDZ domain are involved in the interaction. None of the recombinantly expressed single or tandem PDZ domain constructs was able to reproduce binding to the full length CAP70 protein. In the interaction ELISA, which uses proteins in solution, the structure of the recombinant proteins may be better preserved. This may explain why binding of DRA to the third PDZ domain of CAP70 was detected in the interaction ELISA but not in the overlay assay using single PDZ domain constructs and only to a minor extent using the tandem PDZ domain constructs. Given that CFTR binds to the first, second, and fourth PDZ domains of CAP70 (*22*), clustering of both transport proteins by binding to one CAP70 molecule is possible.

The expression of CAP70 and DRA, however, is not matched in all segments of the gastrointestinal tract: While DRA expression is very high in rabbit proximal colon and lower in the duodenum and ileum (*6*), CAP70 expression is high in the rabbit small intestine and nearly absent in the proximal colon. We conclude from this that CAP70 apparently is not involved in DRA mediated electroneutral NaCl absorption in the colon simply because it is not expressed there.

Nevertheless CAP70 has been implicated both in NHE3 (*29*) and in CFTR function (*22*). How can this be reconciled?

In the intestine Cl^- and HCO_3^- are involved in both fluid absorption and fluid secretion. In terms of fluid absorption Na^+/H^+ exchange and Cl^-/HCO_3^- exchange work in parallel, resulting in electoneutral NaCl absorption (*15*, *16*). On the basis of colocalization and in vitro binding studies we have proposed a model where cAMP induced inhibition of NHE3 and DRA is mediated by E3KARP in the proximal colon and possibly also the distal ileum (*23*).

The role of chloride and bicarbonate in fluid secretion is more complex. cAMP stimulated chloride and bicarbonate secretion, which is most prominent in alkaline secreting epithelia like the pancreas and the duodenum, is dependent on the functional integrity of the CFTR anion channel and most likely occurs through this channel itself (*1*, *17*, *36*, *37*). Apart from this stimulated anion secretion basal bicarbonate secretion is present in the duodenum and occurs through Cl^-/HCO_3^- exchange, which is net electroneutral. Recently we have identified DRA to be present in human and rabbit duodenum. PAT1, a close relative of DRA belonging to the same gene family (SLC26A), has also been identified in the

duodenum, and the relative contribution of the two anion exchangers to duodenal Cl^-/HCO_3^--exchange is unknown (*7*, *20*). Initially the CFTR anion channel was thought not to account for the observed cAMP stimulated bicarbonate secretion, and it was postulated that Cl^- would leave the cell through CFTR and would then be recirculated through the anion exchanger resulting in net electrogenic bicarbonate secretion (*2*, *18*, *38*, *39*). In the meantime CFTR has been shown to conduct bicarbonate and there is strong evidence that cAMP stimulated HCO_3^- secretion occurs through CFTR and that only basal bicarbonate secretion occurs through the anion exchanger (*40*). These data strongly argue against a direct functional coupling of CFTR and the intestinal anion exchanger(s) in the duodenum.

Nevertheless structural coupling is suggested by the fact that CFTR and DRA can be coimmunoprecipitated from pancreas and transfected HEK293 cells (*20*, *21*). CFTR has also been shown in several systems to stimulate the expression and the activity of DRA and PAT1 (*11*, *19*−*21*). This stimulatory function requires CFTR protein to be present in the plasma membrane. More recently Ko et al. (*21*) showed evidence for the direct interaction of CFTR with DRA mediated by the regulatory (R) domain of CFTR and the highly conserved STAS (sulfate transporter and anti-sigma antagonist) domain of DRA. At least when CFTR and DRA expression is low this interaction is also dependent on the C-terminal PDZ interaction motif of CFTR. Thus it has been concluded that CFTR and DRA may be linked by one or several PDZ adapter proteins (*21*).

Because of its restricted expression in the small intestine and its interaction with transport proteins that serve different functions, we suggest that CAP70 mediates a regulatory mechanism specific for the small intestine to these transporters. Given the available data, the most likely function for an interaction of DRA and CFTR with CAP70 in the proximal small bowel would be that this complex is involved in basal and/or stimulated bicarbonate and chloride secretion. But this needs to be proven experimentally. In the distal small bowel CAP70, DRA, and both NHE3 and CFTR are expressed (*6*, *41*, *42*). Therefore it is difficult to speculate about the composition of macromolecular complexes in this segment of the gastrointestinal tract. In addition DRA and NHE3 are inhibited rather than stimulated by cAMP in the context of electroneutral NaCl absorption (*43*).

CAP70 is closely related to NHERF and E3KARP, which in turn are highly homologous. There is controversy about the exact functional role of these PDZ adapter proteins except that they are present at or near the plasma membrane. For CFTR and NHE3 there is evidence for both a role in targeting (*44*−*46*) and a role in the regulation of proteins already present in the plasma membrane (*22*, *47*−*50*). CAP70 interacts with a number of transporters and nontransport proteins (*22*, *26*, *29*, *51*, *52*). The functional consequences of these interactions have as yet only partially been characterized. In vitro the interaction of CFTR with CAP70 induces dimer formation and increases CFTR activity (*22*). In transfected HEK293 cells the interaction of URAT1 with PDZK1, the human orthologue of CAP70, leads to an increased URAT1 cell surface expression and also to an increased URAT1 activity, although the quantitative changes did not match exactly (*51*). A CAP70 knock-out mouse does not have a gross anatomical phenotype but displays a defect in lipoprotein metabolism because of reduced expression of the scavenger receptor class B type I (*53*). Under a high phosphate diet the knock-out animals also show decreased NaP_i-IIa expression and increased renal phosphate excretion compared to the wild-type animals (*54*). Interestingly the expression of PAT1, which mediates Cl^-/formate exchange in the proximal tubule, was also reduced under the high phosphate diet in the knock-out animals but not in the wild-type animals (*54*). Thus the function of CAP70 may be subtle and it will be interesting to study its function in the small bowel, where its expression suggests a specialized function. Thus further studies will have to address the composition of the multiprotein complexes, their localization along the villi, and the functional aspects of plasma membrane targeting and/or regulation of protein complexes present in the membrane.

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