Downregulated in adenoma gene encodes a chloride transporter defective in congenital chloride diarrhea

Moseley, Richard H., Pia Höglund, Gary D. Wu, Debra G. Silberg, Siru Haila, Albert de la Chapelle, Christer Holmberg, and Juhha Kere. Downregulated in adenoma gene encodes a chloride transporter defective in congenital chloride diarrhea. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G185–G192, 1999.—Congenital chloride diarrhea (CLD) is a recessively inherited disorder characterized by massive loss of chloride in stool. We previously identified mutations in the downregulated in adenoma (DRA) gene in patients with CLD and demonstrated that DRA encodes an intestine-specific sulfate transporter. To determine whether DRA is an intestine chloride transporter and how mutations affect transport, Xenopus oocytes were injected with wild-type and mutagenized DRA cRNA and uptake of Cl\textsuperscript{−} and SO\textsubscript{4}\textsuperscript{2−} was assayed. Both Cl\textsuperscript{−} and SO\textsubscript{4}\textsuperscript{2−} were transported by wild-type DRA and an outwardly directed pH gradient stimulated Cl\textsuperscript{−} uptake, consistent with Cl\textsuperscript{−}/OH\textsuperscript{−} exchange. Among three mutants, C307W transported both anions as effectively as wild-type, whereas transport activity was lost in V317del and the double mutant identified in 32 of 32 Finnish CLD patients. We conclude that DRA is a chloride transporter defective in CLD and that V317del is a functional mutation and C307W a silent polymorphism.

Xenopus oocytes; intestine; sulfate; genetic disorders

As a first step toward identification of the CLD gene, the CLD locus was mapped by linkage analysis to chromosome 7q31 adjacent to the cystic fibrosis transmembrane regulator (CFTR) gene and four chromosomal bands away from the Cl\textsuperscript{−}/HCO\textsubscript{3} exchange AE2 (15). Further genetic and physical mapping implicated the downregulated in adenoma (DRA) gene as a positional candidate for CLD (9, 11). The protein product of the DRA gene is a membrane glycoprotein with 10–14 predicted transmembrane domains (4). The DRA protein is highly homologous to a family of sulfate transport proteins (3, 8, 22) and functional studies in Xenopus laevis oocytes have previously confirmed its sulfate transport activity (23, 27). Two sequence changes of DRA, a three-base deletion (GGT) at nucleotides 951–953 that predicts a loss of a valine (V317del) and a T to G transversion at position 921 that predicts an amino acid substitution of tryptophan for cysteine (C307W), were identified in all members of a group of 32 Finnish patients with CLD (10). All 43 parents studied were heterozygous for these two sequence changes; of 32 healthy siblings of CLD patients, 23 were heterozygous for V317del and 9 were homozygous for the wild-type allele (10). Three carriers of the V317del sequence change were identified in an examination of 436 control individuals, all of whom resided in the Eastern part of Finland where the disease is prevalent due to a founder effect (10, 11). However, the presence of two healthy homozygous individuals with the C307W sequence change, the greater than 10-fold population frequency of heterozygotes with the C307W sequence change compared with the overall predicted frequency of carriers of CLD in Finland, and the lack of a CLD-associated geographical distribution for the C307W sequence change all suggest that the C307W sequence change is not disease causing but represents instead a functionally neutral polymorphism (10).

In this study, using functional expression in Xenopus oocytes, we have examined the capacity of the DRA protein to transport chloride as well as sulfate. In addition, we have determined the effect of three sequence changes, V317del, C307W, and V317del/C307W, found in patients affected with CLD on chloride and sulfate transport. Our results demonstrate that the DRA protein functions as a chloride transporter and confirms the prediction, based on the distribution of the three sequence changes in different subpopulations, that V317del is a disease-causing mutation and...
the C307W sequence change is a silent polymorphism. Chloride transport mediated by the DRA protein exhibits features of a Cl\(^{-}\)/OH\(^{-}\) exchanger. The characterization of the DRA protein as a Cl\(^{-}\)/OH\(^{-}\) exchanger or a Cl\(^{-}\)/HCO\(_3\) exchanger, however, requires further investigation.

**METHODS**

**Mutagenesis.** A full-length DRA cDNA (27) inserted in the mammalian expression vector pcMV5 was used as the template for mutagenesis by the ExSite PCR-based site directed mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer’s instructions. The primers used to obtain the different mutants were as follows: C307W/alone, GACTTTTAAAAACAGGTTTAAAG and CACACCCCCCTTGGATTTC and ACAGCCACTTTAAGCTTTTTT; and C307W/V317del combined, TGGGGACATGAATCCTGGATTTC and ACAGCCACTTTAAGCTTTTTT. Mutated clones were identified among nonmutated clones by PCR, Tsp45I digestion, and electrophoresis in 6% polyacrylamide gels, as described (10). The mutated constructs were verified by sequencing the entire coding region.

**In vitro transcription of cDNA.** Wild-type and mutated DRA cDNAs (5 µg) were released from pcMV5 by digestion with Kpn I and Xba I, recloned into Bluescript KS- (Stratagene) and verified again by sequencing. The plasmids were then amplified in DH5\(\alpha\) and purified by alkaline lysis followed by two successive bandings in cesium chloride gradients (21). Each plasmid was linearized with Xba I followed by phenol-chloroform extraction and ethanol precipitation. Capped cRNA was synthesized from the T3 promoter using the Promega RibomAX large scale RNA production kit (Stratagene, La Jolla, CA) according to manufacturer’s instructions. The primers used to obtain the different mutants were as follows: C307W/alone, GACTTTTAAAAACAGGTTTAAAG and CACACCCCCCTTGGATTTC and ACAGCCACTTTAAGCTTTTTT; and C307W/V317del combined, TGGGGACATGAATCCTGGATTTC and ACAGCCACTTTAAGCTTTTTT.

**Oocyte isolation and injection.** Mature Xenopus laevis females were purchased from Xenopus I (Ann Arbor, MI). Frogs were anesthetized by immersion for 15 min in ice-cold water containing 0.3% 3-aminobenzoic acid ethyl ester. Oocytes were removed and incubated at room temperature for 3 h in Ca\(^{2+}\)/Mg\(^{2+}\)-free modified Barth’s solution containing (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO\(_3\), 0.82 MgSO\(_4\), and 10 HEPES-NaOH (pH 7.4) supplemented with 2 mg/ml collagenase (Life Technologies), and stage V and VI oocytes were selected. After overnight incubation at 18°C in modified Barth’s solution (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO\(_3\), 0.82 MgSO\(_4\), 0.33 Ca(NO\(_3\))\(_2\), 0.41 CaCl\(_2\), and 10 HEPES-NaOH (pH 7.4), supplemented with penicillin (500 U/ml) and streptomycin (100 µg/ml), healthy oocytes were injected with 500 pg of cRNA derived from in vitro transcription of wild-type and mutant DRA cDNAs by T7 bacteriophage RNA polymerase (Stratagene). For chloride uptake studies oocytes were incubated for 15 min in a chloride-free uptake solution (in mM, 100 sodium gluconate, 2 potassium gluconate, 1 calcium gluconate, 1 magnesium gluconate, 10 HEPES-Tris, pH 7.5). The oocytes were then incubated in 0.5–1 ml uptake solution containing either 1 mM K\(_2\)SO\(_4\) (20 µCi/ml) or 1 mM Na\(_36\)Cl (5 µCi/ml) for the designated time intervals. Uptake was stopped by washing the oocytes three times with ice-cold uptake solution containing either 5 mM K\(_2\)SO\(_4\) or 5 mM NaCl. Individual oocytes were then transferred to scintillation vials and dissolved in 0.5 ml of 10% SDS, and after addition of scintillation fluid, oocyte-associated radioactivity was determined in a Beckman LS 1801 liquid scintillation counter.

**RESULTS**

In the initial set of experiments cRNAs derived from in vitro transcription of wild-type DRA and two of the mutated genes (V317del alone and V317del/C307W) were injected into a Xenopus oocyte expression system and chloride transport activity was assayed. As shown in Fig. 1, uptake of 1 mM \(^{36}\)Cl was significantly enhanced in oocytes injected with wild-type DRA cRNA compared with chloride uptake in oocytes injected with DEPC-treated water. Furthermore, as shown in Fig. 2, uptake of 1 mM \(^{36}\)Cl was significantly enhanced in oocytes injected with wild-type DRA cRNA compared with chloride uptake in oocytes injected with either of the two mutant DRA cRNAs. In addition, chloride uptake in oocytes injected with either of the two mutant DRA cRNAs was not significantly different from chloride uptake in oocytes injected with DEPC-treated water. The anion exchange inhibitor DIDS, a potent inhibitor of Cl\(^{-}\)/HCO\(_3\) exchange and HCO\(_3\)-independent SO\(_4\)\(^{-}\)/OH\(^{-}\) exchange in rabbit ileal brush-border membrane vesicles (24, 25) and sulfate uptake in DRA-expressing oocytes was injected with either diethyl pyrocarbonate (DEPC)-treated water (total volume 50 nl). Four days after injection, uptake of 1 mM \(^{36}\)Cl was determined at 25°C in medium containing (in mM) 100 Na gluconate, 2 K gluconate, 1 Ca gluconate, 1 Mg gluconate, and 10 HEPES-Tris, pH 7.5. The uptake values represented means ± SE of 12–30 determinations from 2 separate oocyte preparations. *P < 0.05 and **P < 0.005.
cRNA-injected oocytes (27), significantly reduced chloride uptake in oocytes injected with wild-type DRA cRNA. DIDS also significantly reduced chloride uptake in oocytes injected with DEPC-treated water and the V317del mutant DRA cRNA. This finding may be the result of inhibition of endogenous chloride uptake in Xenopus oocytes. Overall, these results show that, in addition to mediating sulfate transport (27), DRA mediates DIDS-sensitive chloride transport that appears to be defective in the V317del alone and V317del/C307W DRA mutants.

Sulfate transport was assayed as before (27), in oocytes injected with cRNA derived from in vitro transcription of either wild-type DRA, V317del alone, or V317del/C307W mutant DRA cRNA (total volume 50 nl). As shown in Fig. 3, 1-h uptake of 1 mM \(^{35}\)S was significantly enhanced in oocytes injected with wild-type DRA cRNA compared with sulfate uptake in oocytes injected with either of the two mutant DRA cRNAs. In addition, sulfate uptake in oocytes injected with either of the two mutant DRA cRNAs was not significantly different from sulfate uptake in oocytes injected with DEPC-treated water. As previously shown (27), DIDS (1 mM) reduced sulfate uptake in oocytes injected with wild-type DRA cRNA without affecting sulfate uptake in oocytes injected with either the V317del mutant DRA cRNA (P = 0.731) or DEPC-treated water (P = 0.612). These results confirm previous findings (27) that DRA mediates DIDS-sensitive sulfate transport and demonstrate that sulfate transport, like chloride transport, appears to be defective in the V317del alone and V317del/C307W DRA mutants.

In separate experiments, cRNA derived from in vitro transcription of wild-type DRA and the mutagenized construct, C307W alone, was injected into a Xenopus oocyte expression system and chloride transport activity was assayed. As shown in Fig. 4A, 1-h uptake of 1 mM \(^{36}\)Cl was not significantly different in oocytes injected with wild-type DRA cRNA compared with chloride uptake in oocytes injected with the C307W mutant DRA cRNA. However, chloride uptake in oocytes injected with either wild-type DRA cRNA or the C307W mutant DRA cRNA was significantly enhanced over chloride uptake in oocytes injected with DEPC-treated water. The differences in absolute uptake values for chloride uptake between this set of experiments and those shown in Figs. 1 and 2 are most likely a reflection of the intrinsic variability in oocyte function between isolations. These results, demonstrating that chloride transport mediated by the C307W mutant DRA is similar to wild-type DRA, are consistent with genetic data that argue against this T to G transversion as a disease-causing mutation (10).

As shown in Fig. 4B, 1-h uptake of 1 mM \(^{35}\)SO\(_4\) was not significantly different in oocytes injected with wild-type DRA cRNA compared with sulfate uptake in oocytes injected with the C307W mutant DRA cRNA. However, sulfate uptake in oocytes injected with either wild-type DRA cRNA or the C307W mutant DRA cRNA was significantly higher than in DEPC-treated water. These results are consistent with genetic data that argue against this T to G transversion as a disease-causing mutation (10).
was significantly enhanced over sulfate uptake in oocytes injected with DEPC-treated water. These results demonstrate that DRA can transport both sulfate and chloride. Therefore, the effect of increasing concentrations of chloride on sulfate uptake in oocytes injected with either wild-type DRA cRNA, V317del mutant DRA cRNA, or DEPC-treated water was examined. As illustrated in Fig. 5, chloride caused a concentration-dependent decrease in the 1-h uptake of 1 mM $^{35}$SO$_4$ in oocytes injected with wild-type DRA cRNA. In this set of experiments, at high chloride concentrations, sulfate uptake in oocytes injected with wild-type DRA cRNA was not significantly different from sulfate uptake in oocytes injected with DEPC-treated water. Sulfate uptake in the absence of chloride (and in the presence of 10 mM chloride) in oocytes injected with V317del mutant DRA cRNA was not significantly different from sulfate uptake in oocytes injected with DEPC-treated water.

As an initial step to characterize the mechanism for chloride transport mediated by the DRA protein, the effect of an outwardly directed pH gradient on chloride uptake in oocytes injected with either wild-type DRA cRNA, C307W, V317del alone, V317del/C307W mutant DRA cRNA, or DEPC-treated water was examined. As illustrated in Fig. 6, 1-h uptake of 1 mM $^{36}$Cl was significantly greater in oocytes injected with wild-type DRA and C307W cRNA compared with chloride uptake in oocytes injected with V317del alone and V317del/C307W mutant DRA cRNA. The addition of DIDS significantly inhibited pH gradient-stimulated chloride uptake in oocytes injected with wild-type DRA and C307W cRNA. Of note, 1-h uptake of 1 mM $^{36}$Cl was...
significantly greater ($P < 0.005$) in oocytes injected with V317del alone and V317del/C307W mutant DRA cRNA compared with chloride uptake in oocytes injected with DEPC-treated water and the addition of bumetanide had no effect on chloride uptake in these oocytes. These results demonstrate that the DRA protein exhibits features similar to apical Cl$^-$/OH$^-$ exchange in rat colon.

DISCUSSION

Although intestinal perfusion studies suggested that the chloride malabsorption observed in CLD was the result of a defect in Cl$^-$/HCO$_3^-$ exchange in both the ileum and the colon, mapping of the CLD locus by linkage analysis to chromosome 7q31 adjacent to the CFTR gene and four chromosomal bands away from the Cl$^-$/HCO$_3^-$ exchanger AE2 (15), implicated other intestinal chloride transport processes in this disorder. Further genetic and physical mapping implicated the DRA gene as a positional candidate for CLD (9, 11). The identification of three different DRA sequence changes, including the double-sequence change in 32 of 32 Finnish patients in homozygous form and in heterozygous form in all 43 parents (10) provided further evidence that DRA is involved in intestinal chloride transport and that mutations of DRA result in CLD. In this study we have demonstrated that in addition to sulfate transport activity (27) DRA mediates chloride transport with features suggestive of Cl$^-}$/OH$^-$ exchange. Furthermore, the V317del mutation of DRA results in the loss of both chloride and sulfate transport activity, whereas the C307W sequence change appears to be a functionally silent polymorphism.

V317del/C307W mutant DRA cRNA compared with chloride uptake in oocytes injected with DEPC-treated water and the addition of bumetanide had no effect on chloride uptake in these oocytes. These results demonstrate that the DRA protein exhibits features similar to apical Cl$^-$/OH$^-$ exchange in rat colon.

**Fig. 6.** Effect of an outwardly directed pH gradient on chloride uptake in Xenopus oocytes. Oocytes were injected with either DEPC-treated water, 500 pg WT DRA cRNA, C307W, V317del alone, or V317del/C307W mutant DRA cRNA (total volume 50 nl). Five days after injection, 1-h uptake of 1 mM $^{36}$Cl was determined at 25°C in medium containing (in mM) 100 Na gluconate, 2 K gluconate, 1 Ca gluconate, 1 Mg gluconate, 3 Tris, 1.4 mM HEPES, and 9 mM MES, pH 6.0, in presence or absence of 1 mM DIDS. Uptake values represent means ± SE of 28–52 determinations from 4 separate oocyte preparations. **$P < 0.005$ for uptake values relative to WT DRA cRNA.

**Fig. 7.** Effect of bumetanide (B) on chloride uptake in Xenopus oocytes. Oocytes were injected with either DEPC-treated water, 500 pg WT DRA cRNA, C307W, V317del alone, or V317del/C307W mutant DRA cRNA (total volume 50 nl). Five days after injection, 1-h uptake of 1 mM $^{36}$Cl was determined at 25°C in medium containing (in mM) 100 Na gluconate, 2 K gluconate, 1 Ca gluconate, 1 Mg gluconate, 3 Tris, 1.4 HEPES, and 9 MES, pH 6.0, in presence or absence of 1 mM bumetanide. Uptake values represent means ± SE of 25–34 determinations from 4 separate oocyte preparations. *$P < 0.05$ for uptake values relative to WT DRA cRNA.
The protein product of the DRA gene is a membrane glycoprotein with 10–14 predicted transmembrane domains (4). Protein expression is restricted to the columnar epithelial cells, particularly to the apical (brush-border) membrane (4). As shown in Fig. 8, three models of the secondary structure of the DRA protein have been proposed that vary in the number of transmembrane domains. In the top model, the V317del mutation lies in the fourth intracellular domain of the DRA protein (4, 10). In the other two models the V317del mutation lies in the fourth extracellular domain of the DRA protein. Although the V317del mutation does not affect the transcriptional activity of the gene (10), it remains to be determined whether this mutation results in the insertion of functionally defective protein into the apical membrane of absorptive enterocytes or whether defective chloride and sulfate transport represents a trafficking defect similar to that recently described for the intestinal Na+-dependent glucose transporter (19). Results depicted in Figs. 6 and 7 demonstrating that chloride uptake into oocytes injected with ΔV317 alone and ΔV317/C307W mutant DRA cRNA is significantly greater than chloride uptake in oocytes injected with DEPC-treated water and is unaffected by the inhibitors DIDS and bumetanide suggest that a functionally defective protein is inserted into the membrane that nonetheless can support the movement of chloride down an electrochemical gradient. The C307W sequence change might also be expected to affect the secondary structure of the DRA protein through the loss of an intramolecular disulfide bond. However, cysteine 307 is not a conserved amino acid.

Fig. 8. Predicted secondary structures of DRA protein. Localization of the observed amino acid and nucleotide alterations are indicated. Potential N-linked glycosylation sites are also indicated by G (adapted in part from Refs. 4 and 10).
acid in either of the sulfate transporters encoded by the Sat-1 and DTDST genes (3, 8).

We have previously identified DRA as an intestine-specific sulfate transporter that is sensitive to DIDS and oxalate (27). A Cl⁻/OH⁻ antiport has been identified in isolated rat and rabbit brush-border membrane vesicles capable of exchanging luminal Cl⁻ for intracellular OH⁻, Cl⁻, and/or HCO₃⁻ (16, 17). However, sulfate did not inhibit pH and HCO₃⁻ gradient-stimulated Cl⁻ uptake (16), suggesting that sulfate is not a substrate for this ileal Cl⁻/OH⁻ (HCO₃⁻) exchanger. In addition, electroneutral, HCO₃⁻-independent, Cl⁻/OH⁻ exchange has been identified in ileal brush-border membrane vesicles and, of note, a defect in this transport process in CLD was previously proposed (30). The effect of sulfate on Cl⁻ uptake was not, however, examined in these vesicle transport studies. A SO₄²⁻/OH⁻ exchanger has also been identified in rabbit ileal brush-border membrane vesicles (24). In contrast to the lack of an effect of sulfate on pH gradient-stimulated Cl⁻ uptake (24), Cl⁻ and oxalate both significantly inhibited pH gradient-stimulated SO₄²⁻ uptake (25). Cl⁻/HCO₃⁻ and Cl⁻/OH⁻ exchange have also both been described in apical membrane vesicles of rat distal colon (20). In this study, HCO₃⁻ gradient-stimulated chloride uptake was inhibited by 1 mM bumetanide by only 13%, whereas OH⁻ gradient-stimulated chloride uptake was inhibited by 43% (20). Oxalate (1 mM) also significantly inhibited OH⁻ gradient-stimulated chloride uptake (20). Therefore, the results of this and previous studies (27) strongly suggest that DRA is an apical Cl⁻ (SO₄²⁻)/OH⁻ exchanger in the intestine and that the defect underlying CLD resides in this transporter. Nevertheless, additional studies are required to determine whether DRA functions as an intestinal Cl⁻ (SO₄²⁻)/HCO₃⁻ exchanger.

DRA expression is much higher in the colon than the ileum (27). Because the colon is the major site of water absorption, this finding supports the hypothesis that a mutation in the DRA protein results in CLD. Physiologically, these results suggest that in the normal distal ileum and colon chloride is preferentially absorbed proximally. Sulfate absorption is increased in the distal colon where chloride concentrations are low due to efficient proximal absorption. Phenotypically, the defect in intestinal sulfate absorption, however, does not result in distinct clinical manifestations.

DRA was originally identified as a gene that was expressed in normal colonic tissue but was significantly decreased or absent in adenomas and adenocarcinomas (26), an expression profile that has been recently confirmed using the method of serial analysis of gene expression in normal and neoplastic cells (31). This expression profile initially led to speculation that it might represent a tumor suppressor gene involved in colon carcinogenesis (26). With the demonstration that DRA is an intestine-specific anion transporter, it remains unclear whether loss of transport function may be involved in the development of the malignant phenotype or is an epiphenomenon. Intracellular pH (pHᵢ) regulation appears to be involved in cellular growth and cell division (7) and the higher pHᵢ, for example, in the SW-620 human colon carcinoma cell line is accounted for by the absence of Cl⁻/OH⁻ (HCO₃⁻) exchange (2). However, there is no established association between CLD and colonic adenomas and adenocarcinomas. Furthermore, mortality in patients affected with CLD has been linked only to inadequate replacement therapy (13). Indeed, the loss of DRA expression, normally restricted to the differentiated surface epithelium of the colon, in adenomas and adenocarcinomas may simply reflect the loss of a differentiated phenotype during the process of neoplasia. The loss of DRA expression in a tubular adenoma in a Finnish CLD patient (10) is consistent with this hypothesis.

DRA is closely related to the genes encoding the diastrophic dysplasia sulfate transporter (DTDST) (8) and the rat canalicular SO₄/HCO₃ exchanger Sat-1 (3). Injection of rat and human DTDST cDNA into Xenopus oocytes was recently shown to induce Na⁺-independent sulfate transport that was inhibited by extracellular chloride and bicarbonate (23). In contrast, Sat-1-directed sulfate uptake was stimulated by extracellular chloride and inhibited by bicarbonate. Although interpreted to suggest that DTDST functions as a sulfate/chloride antiporter, these results are also compatible with the notion that DTDST is a Cl⁻ (SO₄²⁻)/OH⁻ (HCO₃⁻) exchanger. Thus this family of genes may all encode anion exchangers.

In summary, we have demonstrated that the DRA gene product functions not only as a sulfate transporter but also confers chloride transport activity, with features consistent with Cl⁻/OH⁻ exchange. A mutation that is present in homozygous form in all of 32 Finnish patients affected with CLD abolishes both chloride and sulfate transport, whereas anion transport is unaffected by another sequence change that is present in homozygous form in both CLD patients and healthy controls. These findings functionally confirm that a mutated DRA gene is responsible for the severe loss of chloride in stool that characterizes this disorder. The clinical picture of CLD, identification of mutations of DRA in CLD patients, and the present results establish the DRA protein as the anion exchanger that physiologically is most likely responsible for absorbing greater than 90 mmol/l of chloride from the intestinal lumen along the length of the distal ileum and colon. An understanding of the molecular background of a rare disease has therefore made it possible to define a role for one of the many intestinal transporters.

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