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Mutations in the chloride-bicarbonate exchanger gene *AE1* cause autosomal dominant but not autosomal recessive distal renal tubular acidosis

F. E. KARET[a], F. J. GAINZA[b], A. Z. GYÖRY[c], R. J. UNWIN[d], O. WRONG[d], M. J. A. TANNER[e], A. NAYIR[f], H. ALPAY[f], F. SANTOS[g], S. A. HULTON[h], A. BAKKALOGLU[i], S. OZEN[i], M. J. CUNNINGHAM[j], A. DI PIETRO[k], W. G. WALKER[l], AND R. P. LIFTON[a, m]

[a]Howard Hughes Medical Institute, Departments of Medicine and Genetics, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT 06510; [b]Hospital de Cruces, Barakaldo-Bizkaia, Basque Country, Spain; [c]University of Sydney, Royal North Shore Hospital, St. Leonards, N.S.W., Australia; [d]Institute of Urology and Nephrology, University College London, London W1N 8AA, U.K.; [e]Department of Biochemistry, University of Bristol, Bristol BS8 LTD, U.K.; [f]Department of Pediatric Nephrology, University of Istanbul, Istanbul, Turkey; [g]Division of Pediatric Nephrology, Universidad de Oviedo, Oviedo, Spain; [h]Department of Nephrology, Birmingham Children's Hospital, Birmingham B16 8ET, U.K.; [i]Department of Pediatric Nephrology, Children's Hospital, Hacettepe University, Ankara, Turkey; [j]Massachusetts Eye & Ear Hospital, Harvard University, Boston, MA 02114; [k]Servizio Ospedaleria di Rilievo Nazionale, Napoli, Italy; and [l]Johns Hopkins University, Baltimore, MD 21205

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ABSTRACT **Primary distal renal tubular acidosis (dRTA) is characterized by reduced ability to acidify urine, variable hyperchloremic hypokalemic metabolic acidosis, nephrocalcinosis, and nephrolithiasis. Kindreds showing either autosomal dominant or recessive transmission are described. Mutations in the chloride-bicarbonate exchanger *AE1* have recently been reported in four autosomal dominant dRTA kindreds, three of these altering codon Arg589. We have screened 26 kindreds with primary dRTA for mutations in *AE1*. Inheritance was autosomal recessive in seventeen kindreds, autosomal dominant in one, and uncertain due to unknown parental phenotype or sporadic disease in eight kindreds. No mutations in *AE1* were detected in any of the autosomal recessive kindreds, and analysis of linkage showed no evidence of linkage of recessive dRTA to *AE1*. In contrast, heterozygous mutations in *AE1* were identified in the one known dominant dRTA kindred, in one sporadic case, and one kindred with two affected brothers. In the dominant kindred, the mutation Arg-589/Ser cosegregated with dRTA in the extended pedigree. An Arg-589/His mutation in the sporadic case proved to be a *de novo* mutation. In the third kindred, affected brothers both have an intragenic 13-bp duplication resulting in deletion of the last 11 amino acids of AE1. These mutations were not detected in 80 alleles from unrelated normal individuals. These findings underscore the key role of Arg-589 and the C terminus in normal AE1 function, and indicate that while mutations in *AE1* cause autosomal dominant dRTA, defects in this gene are not responsible for recessive disease.**

The maintenance of body fluid pH within a narrow range is critical for a wide variety of essential biochemical and metabolic functions. The kidney plays a key role in this homeostasis under normal circumstances, owing to its ability to vary bicarbonate reclamation and net acid excretion over a wide range. In the renal tubular acidoses (RTAs), however, acid-base balance becomes deranged either because of inability to secrete acid in the distal nephron or because of proximal bicarbonate loss (1).

Primary distal RTA (dRTA) is characterized by the failure of the kidney to produce an appropriately acid urine in the presence of systemic metabolic acidosis or after acid loading, due to failure of hydrogen ion secretion in the distal nephron (2). This results in hyperchloremic metabolic acidosis of varying severity. The condition is usually accompanied by nephrocalcinosis or nephrolithiasis. Other findings include hypokalemia and normal serum calcium and phosphate levels, though osteomalacia or rickets may supervene in untreated cases. Alkali replacement serves to reverse most of the biochemical abnormalities.

Both autosomal dominant and autsomal recessive inheritance patterns may be observed in different kindreds with primary dRTA, and the spectrum of clinical severity is wide. Some patients with autosomal dominant dRTA remain asymptomatic until adolescence or adulthood, whereas others, and those with recessive disease, may be severely affected in infancy, with impaired growth and early nephrocalcinosis causing eventual renal insufficiency. Many patients with recessive dRTA also have sensorineural deafness (3).

The pathophysiologic basis of primary dRTA has remained elusive, many investigators hypothesizing that defective apical proton transport may be the underlying mechanism. In the alpha-intercalated cells of the collecting duct (Fig. 1), urinary acidification involves a combination of energy-dependent proton secretion across the apical surface, mediated by both hydrogen and hydrogen-potassium ATPases (4), and basolateral chloride-bicarbonate exchange, which serves to transport bicarbonate back into the blood (5). The latter occurs via the band-3 protein AE1, a 911-amino acid membrane protein (6). This molecule is thought to have 14 membrane-spanning regions between residues 404 and 882, across which anion exchange occurs, flanked by intracellular N- and C-terminal domains (7).

AE1 is also present in red cells, where it contributes to cytoskeletal structure through N-terminal interactions with ankyrin and protein 4.2 (8). A variety of mutations in these interactive domains have been identified as the cause of hereditary spherocytosis and Southeast Asian ovalocytosis (8, 9). *AE1* is located on chromosome 17q21–22 (10).

Recently, investigation of four kindreds with autosomal dominant dRTA has revealed mutations in *AE1* (11). Strik-

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Abbreviations: RTA, renal tubular acidosis; dRTA, distal RTA.
[m]To whom reprint requests should be addressed at Departments of Medicine and Genetics, Howard Hughes Medical Institute, Yale University School of Medicine, Boyer Center for Molecular Medicine, Room 154, 295 Congress Avenue, New Haven, CT 06510. e-mail: richard_lifton@qm.yale.edu.

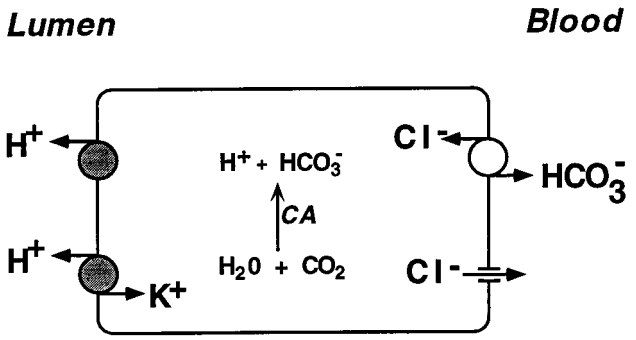

FIG. 1. Proton and bicarbonate transport in the alpha-intercalated cell of the distal tubule. At the apical surface, hydrogen ions produced by the carbonic anhydrase-catalyzed hydration of CO_2 are secreted via the ATP-driven hydrogen pump and hydrogen-potassium exchanger. At the basolateral membrane, bicarbonate is reabsorbed in exchange for chloride via the anion exchanger AE1. Chloride exits the cell through a separate channel. *CA*, carbonic anhydrase.

ingly, a single base change alters the identical residue, Arg-589, in three of the four kindreds. This represents the first evidence that anion rather than cation transport function is at fault in dominant dRTA. It is intriguing to note that *in vitro* expression of the mutations does not appear to result in loss of function. These findings have established the association of *AE1* mutations with dominant dRTA, motivating the present study that sought to extend this mutational finding in dominant disease and to investigate a possible role for *AE1* in recessive dRTA.

MATERIALS AND METHODS

dRTA Kindreds. Kindreds were recruited via ascertainment of affected index cases. Individuals were classified as affected either because they failed to reduce urine pH below 5.3 after oral ammonium chloride loading (12), or because they had inappropriately alkaline urine (pH $>$ 6.4) in the presence of systemic metabolic acidosis. None of these patients had evidence of secondary causes of dRTA. Biochemical analysis included measurement of serum sodium, potassium, calcium, phosphate, and magnesium, as well as measurement of urinary protein. Inheritance patterns were prospectively ascribed as dominant where individuals in successive generations were affected, and as recessive where either there were two or more affected offspring of unaffected parents, or when a single case was the offspring of consanguineous union of unaffected parents. Sporadic cases who were the offspring of unrelated parents, and affected siblings whose parental phenotypes were unavailable, were classified as transmission pattern uncertain. Genomic DNA was prepared from venous blood of members of RTA kindreds by standard procedures as described (13).

Mutation Detection and DNA Sequencing. Molecular variants in *AE1* were sought by analysis of single-strand conformational polymorphism (14). Using genomic DNA of disease family members or controls as template, all coding exons of the renal isoform of *AE1* were individually amplified by PCR by using primers and conditions previously described (15). Amplified products were denatured and analyzed for molecular variants by electrophoresis under two different non-denaturing conditions, as described (16). Identified variants were eluted from gel, reamplified by PCR and the DNA sequence of both strands determined by using the dideoxy termination method on an ABI 377 instrument (Applied Biosystems). All variants were confirmed by independent amplifications from genomic DNA.

Genotyping. For homozygosity mapping and linkage analysis, genotyping of polymorphic loci flanking the location of *AE1*, as indicated in the text, was performed by PCR as described (16). Labeling was either by incorporation using α-^{32}P-dCTP, or by fluorescent end-labeling and analysis on an ABI 377 instrument. All genotypes were scored independently by two investigators blinded to affection status. In family RTA8, nine loci on three other chromosomes were also typed to confirm parental status. Homozygosity mapping was performed using the MAPMAKER/HOMOZ and GENEHUNTER programs (17, 18) run on a Sun Sparcstation 20. Both stringent and conservative models were employed. The former specified a disease gene frequency of 1:1000, 100% penetrance and 0% phenocopy rate; the latter used a gene frequency of 1:200, 95% penetrance and 10% phenocopies.

RESULTS

dRTA Kindreds. As outlined in Fig. 2 and Table 1, 26 kindreds were recruited for study, comprising 40 living sub-

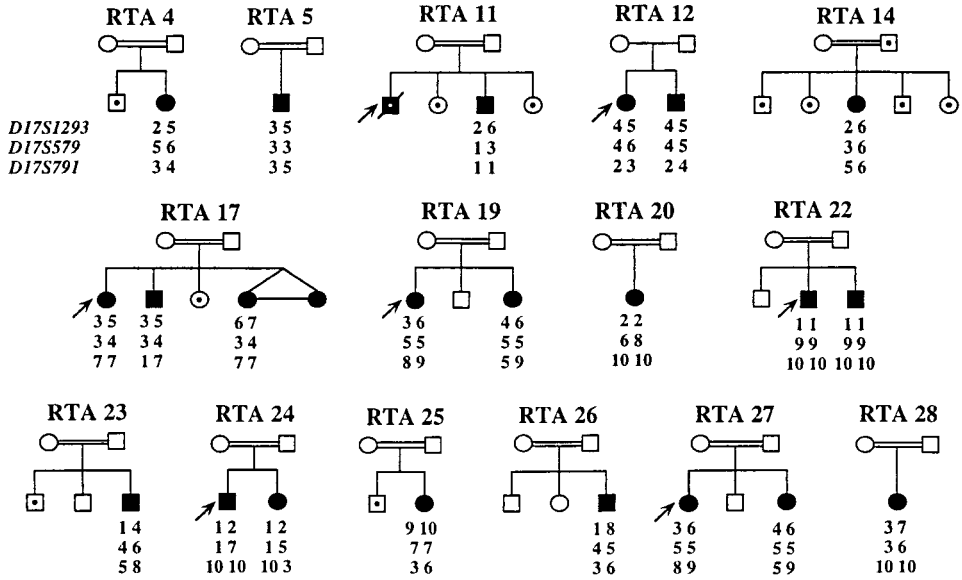

FIG. 2. Structures of autosomal recessive RTA kindreds in which linkage studies were performed. Affected and unaffected subjects are indicated by solid and open symbols, respectively. Dots indicate individuals unavailable for study. Double lines indicate parental consanguinity; all are first cousins except kindreds RTA23 and RTA24 who are second cousins. Genotypes at polymorphic loci closely flanking *AE1* are shown.

Table 1. Selected clinical and biochemical features of dRTA patients

Patient	Age at diagnosis	Deaf	Consang	K	Cl	HCO_3	pH	Nephrocalcinosis
Dominant								
10-1	27 yr	No	No	4.4	111	19.5	7.35	No
10-2	25 yr	No		4.6	114	19.4	7.37	No
10-3	10 yr	No		4.2	108	19.5	7.29	No
10-4	10 yr	No		3.7	109	19.5	7.29	No
10-5	10 yr	No		3.7	108	17.4	7.30	No
10-7	17 yr	No		3.4	110	20.1	7.38	No
Recessive								
2-1	4 yr	Yes	No	3.1	110	15.5	NA	Yes
3-1	6 wk	No	No	2.2	125	9.7	7.22	Yes
4-1	3 wk	No	Yes	2.4	110	5.5	7.1	Yes
5-1	1 yr	No	Yes	3.1	106	11.0	7.12	Yes
11-1	5 yr	No	Yes	1.58	108	12.0	7.2	Yes
12-1	2 mo	Yes	No	2.6	140	NA	7.30	Yes
12-2	5 mo	Yes		NA	NA	NA	NA	NA
14-1	6 yr	No	Yes	3.5	105	13.0	7.2	Yes
17-1	1 wk	No	Yes	2.7	116	12.0	7.25	Yes
17-2	3 wk	No		4.1	NA	12.0	NA	Yes
17-3*	1 wk	No		4.5	NA	13.0	NA	Yes
19-1	3 mo	Yes	Yes	2.6	108	8.4	7.2	Yes
19-2	11 mo	Yes		2.8	102	6.7	7.12	Yes
20-1	2 mo	No	Yes	2.3	119	4.8	7.07	Yes
22-1	4 mo	No	Yes	2.7	104	12.0	7.2	Yes
22-2	6 mo	No		2.34	104	13.6	7.21	Yes
23-1	3 mo	No	Yes	2.8	NA	12.0	7.1	Yes
24-1	2 yr	Yes	Yes	2.8	109	6.3	7.1	Yes
24-2	4 yr	Yes		2.8	110	11.0	7.2	Yes
25-1	2 mo	Yes	Yes	2.4	114	15.0	7.3	Yes
26-1	1 mo	Yes	Yes	2.1	102	10.0	7.1	Yes
27-1	3 mo	No	Yes	2.8	102	12.0	7.16	Yes
27-2	5 yr	No		2.2	106	14.0	7.18	Yes
28-1	1 yr	No	Yes	2.77	94	14.0	7.23	Yes
Transmission uncertain								
6-1	25 yr	No	No	3.3	NA	19.5	NA	Yes
6-2	37 yr	No		3.3	NA	17.8	NA	Yes
7-1	5 mo	Yes	No	3.0	124	14.8	7.26	Yes
8-1	12 yr	No	No	3.1	NA	10.3	7.21	Yes
13-1	9 yr	No	No	2.9	116	12.0	7.3	Yes
15-1	1 mo	No	No	3.2	113	13.4	7.24	Yes
16-1	19 mo	No	No	4.5	115	11.1	7.23	No
18-1	3 mo	Yes	No	2.2	119	13.0	7.26	Yes
29-1	6 mo	No	No	3.2	104	11.0	7.12	Yes

Consang, consanguineous union of related parents (see Fig. 2); NA, not available. Normal ranges: plasma K, 3.5–5.0 mmol/liter; Cl, 94–104 mmol/liter, HCO_3, 20–30 mmol/liter; arterial pH, 7.36–7.44. *Values for elder of monozygotic twins.

jects. Four kindreds had previously been reported (3, refs. 19–21). Of these, one was autosomal dominant (RTA10). Seventeen kindreds were prospectively classified as recessive. Among these, affected individuals were offspring of consanguineous union in 14 (12 first-cousin and 2 second-cousin marriages), and there were two or more affected offspring of unaffected parents in the other three (Fig. 2). Affected members of eight kindreds were classified as transmission uncertain; seven were single affected offspring of unaffected unrelated parents, and one kindred (RTA6) comprised two affected brothers with deceased parents.

Clinical Characteristics. Among the recessive kindreds, nineteen of the 25 patients were diagnosed at one year of age or less, and the remainder presented at 6 years or younger. All index cases presented either acutely with vomiting and dehydration, or with failure to thrive, or delayed growth. Younger affected siblings were often diagnosed prospectively. All patients with recessive disease were found to have nephrocalcinosis, nephrolithiasis, or both, and several had rickets. Nine of these patients from six families also had bilateral sensorineural deafness confirmed by audiometry.

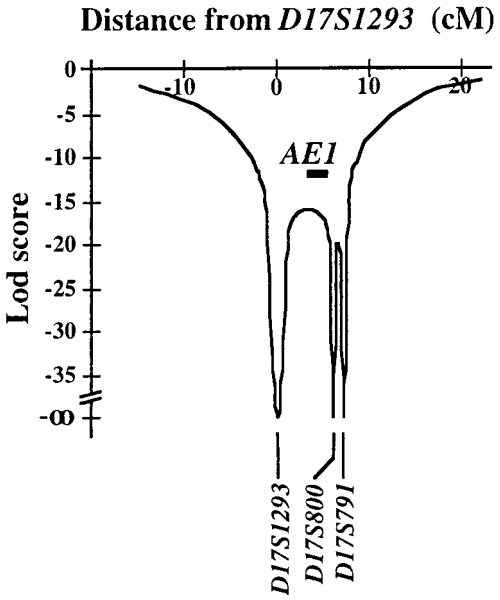

FIG. 3. Homozygosity mapping in the 14 consanguineous recessive kindreds. The result of multipoint comparison of affectation status with genotypes at polymorphic loci tightly linked to *AE1* is shown under the stringent model of linkage. Genetic distances between these loci, and the region within which *AE1* is thought to lie, are displayed.

In contrast, in the one dominant kindred (RTA10), two propositae were diagnosed because of nephrolithiasis at ages 56 and 36 years. Prospective screening identified other affected family members who were all asymptomatic, and most were diagnosed in adulthood. To date, none of the six reported here has had radiological evidence of nephrocalcinosis.

A variety of clinical presentations was observed in the eight kindreds with uncertain transmission pattern. Patients in five kindreds were diagnosed at <2 years of age, and of these, two were deaf. In contrast, the affected brothers in kindred RTA6 presented in adulthood with nephrolithiasis, having previously been asymptomatic. Patient RTA8–1 had impaired growth, acidosis and rickets, and was diagnosed at age 12 years. Nephrocalcinosis was found in all but the affected members of kindreds RTA6 and -16.

Biochemical Findings. At diagnosis, all affected patients either had urine pH > 6.5 in the presence of metabolic acidosis (arterial pH 7.07–7.3), or failed to acidify their urine below pH 5.7 after standard oral ammonium chloride loading, or both. All recessive and six of the eight transmission-uncertain patients had severe metabolic acidosis with arterial pH ≤ 7.3 and plasma HCO_3 ≤ 15.5 mmol/liter (Table 1). In contrast, three of the six patients in the dominant kindred had milder acidosis, maintaining blood pH within the normal range. All six, together with the affected brothers in kindred RTA6, had plasma $HC0_3$ ≥ 17.4 mmol/liter. All index cases were hypokalemic and hyperchloremic, and after correction of dehydration where necessary, all patients had normal serum sodium, calcium, magnesium, and phosphate levels. In all cases, alkali replacement resulted in improvement in biochemical parameters. Creatinine clearance remains normal in all but patients 2–1 (who has end-stage renal disease), 6–1, 6–2, and 8–1 (serum creatinine 1.8, 1.6, and 3.1 mg dl^{-1}, respectively). No patients had significant proteinuria.

Absence of Linkage and Mutation in Recessive dRTA. Evidence for linkage in recessive kindreds was sought by genotyping polymorphic loci tightly linked to *AE1* on chromosome 17q21–22. Loci *D17S1293, D17S579*, and *D17S791*, all

of which have heterozygosities of over 84% (22), span a genetic distance of 7cM. *AE1* lies within the 2-cM region centromeric to *D17S579*. Family structures and genotypes are shown in Fig. 2. In the 14 consanguineous kindreds, linkage was assessed by homozygosity mapping (23, 17). As shown in Fig. 2, genotypes of the affected individuals revealed that in only one of these consanguineous kindreds were affected individuals homozygous across the *AE1* interval, not significantly different from the proportion expected under the null hypothesis of no linkage. Analysis of these families yielded a maximum lod score of −15.54 under the stringent model of linkage, between *D17S1293* and *D17S579*, as displayed in Fig. 3. Allowing for locus heterogeneity, increased gene frequency and proportion of phenocopies failed significantly to alter this result, with a maximum lod score of −11.84 under the conservative model. A lod score of −2.0 (odds ratio of 100:1 against linkage) is sufficient to exclude linkage to a region.

In addition, restriction of analysis of linkage to only those kindreds with two or more affected siblings (RTA12, -17, -19, -22, -24, and -27) (ref. 18) also yielded lod scores rejecting linkage, with maximum lod scores across this interval of −5.64 and −4.44 under stringent and conservative models, respectively. In the remaining two outbred recessive kindreds, prior death of one affected sibling precluded inclusion in analysis of linkage.

Moreover, screening of all coding exons in *AE1* by single-strand conformational polymorphism in affected members of all 17 recessive kindreds did not identify any variants that alter the encoded protein.

These data together exclude linkage of autosomal recessive dRTA to *AE1* and indicate that recessive disease is rarely, if ever, caused by mutation in *AE1*.

***AE1* Mutations in Dominant dRTA.** In contrast to these results in recessive kindreds, Fig. 4 illustrates heterozygous mutations altering the encoded AE1 protein that were found in the one dominant kindred (RTA10), in one sporadic case (RTA8–1), and in the kindred with two affected brothers whose parents were deceased (RTA6).

In the dominant kindred RTA10, a single base change results in a missense mutation in which serine is substituted for arginine at codon 589, the identical codon found to be mutated in three of the four previously reported kindreds (11). This mutation cosegregates with the disease in seven members of the family (Fig. 4*a*).

Similarly, the sporadic case in kindred RTA8 also has a mutation in codon 589, in this case substituting a histidine residue (Fig. 4*b*). This mutation is absent in both parents and the unaffected siblings of the index case. Genotyping of 12 polymorphic loci distributed across four chromosomes confirms both mother and father as the biological parents (data not shown). Moreover, one unaffected sister and the index case

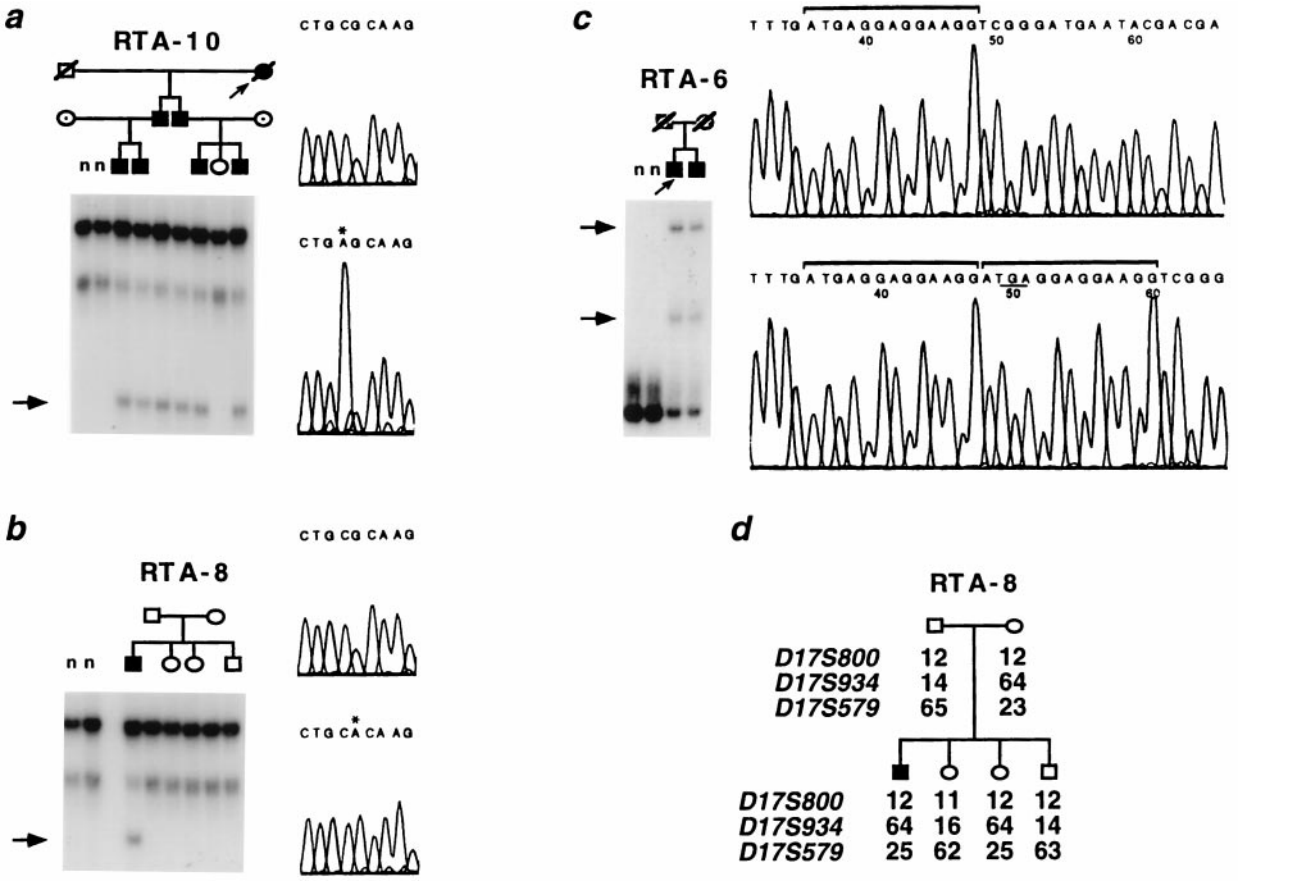

FIG. 4. Mutations in *AE1* in dominant dRTA patients. The structure of each kindred is shown. Affected, unaffected, and phenotype-unknown subjects are indicated by solid, open, and shaded symbols, respectively. *n* = unrelated normal subjects. Below the diagram of each kindred in *a–c* are the results of single-strand conformational polymorphism analysis of exons 14, 14, and 20 of *AE1*, respectively. The corresponding DNA sequence of the sense strand of wild-type (*Upper*) and mutant alleles (*Lower*) are also shown. Arrows indicate variants specific to RTA patients. In *a* and *b*, asterisks above sequences indicate the variant bases in codon Arg-589. (*a*) In kindred RTA10, CGC is changed to AGC (589Ser) in all six affected members. (*b*) In kindred RTA8, only the affected index case shows the *de novo* mutation altering CGC to CAC (589His). (*c*) The bracket shows the 13-bp sequence present in single copy in wild-type sequence, but duplicated in tandem in both affected members of RTA6. The result leads to premature termination at codon 901 (underlined), truncating the protein by 11 amino acids. (*d*) Genotypes and haplotypes of kindred RTA8. Haplotypes flanking *AE1* are shown, which confirmed biological parentage, and in addition show that the index case was identical with an unaffected sister. The results confirm the presence of a *de novo* mutation.

inherit the identical haplotypes flanking *AE1*, yet do not share the *AE1* mutation (Fig. 4*d*). These observations demonstrate that the Arg-589/His mutation in this patient is a *de novo* mutation appearing concordantly with the disease, providing very strong genetic evidence that this mutation causes dRTA.

An *AE1* mutation in exon 20 was also identified in both affected brothers in kindred RTA6 (Fig. 4*c*). Here a 13-bp sequence extending from the second base in codon 896 to the second base in codon 900 is duplicated in tandem. This duplication does not alter the encoded protein through codon 900, but results in a premature termination codon at position 901, truncating the protein by 11 amino acids.

None of these mutations was identified in examination of 80 alleles for each exon from 40 unrelated and unaffected subjects.

DISCUSSION

dRTA was first formally described in the 1930s (24, 25). At that time it was thought to be due to back-diffusion of normally secreted protons across a "leaky" tubular epithelium. Over the next five decades, numerous investigators have demonstrated in animal and human studies that tubular acid secretion itself is abnormal (26). Whether this is a primary or secondary event at the molecular level has, however, remained an enigma. In this report we have extended the recent finding of *AE1* mutations in four dominant dRTA kindreds (11), thus confirming that the autosomal dominant form of this disease is commonly caused by mutations in this gene. In contrast, we have shown by analysis both of linkage and mutation, that mutation in *AE1* rarely if ever causes autosomal recessive dRTA, indicating the presence of at least one additional gene in which mutation causes this phenotype.

We have demonstrated heterozygous mutations in *AE1* in three kindreds: one with definite autosomal dominant dRTA, one with a *de novo* mutation, and one kindred with two affected brothers. These genetic findings are consistent with all three kindreds having autosomal dominant disease. The observation that all of these cases presented at older ages with relatively mild or even absent clinical features is consistent with this classification, there being no previous reports of recessive disease with such mild phenotype. The mutations identified in two kindreds affect the same codon, Arg-589, as in three of the previously reported dominant kindreds (11), while the third mutation is previously undescribed, deleting the last 11 amino acids of AE1. Finding different Arg-589 mutations in different kindreds, as well as the *de novo* mutation, indicates that the high prevalence of these mutations is not attributable to a shared founder mutation.

It is striking that Arg-589 mutations have now been found in five of the seven dominant kindreds reported (two of three described here and three of four in ref. 11), supporting the importance of this residue in the normal acidification process. Arg-589 lies at the intracellular border of the sixth transmembrane domain of the protein, adjacent to Lys-590; this latter residue is the target for the specific AE1 inhibitor phenyl isothiocyanate (27). These basic residues are conserved in all the known vertebrate anion exchanger isoforms and are thought to form part of the site of intracellular anion binding (28). However, expression of the recently identified Arg-589 mutants in *Xenopus* oocytes did not lead to loss of chloride transport (11), leading the authors to speculate that this and other *AE1* mutations causing dRTA do not simply result in loss of function. Instead they might, for example, alter the specificity of the usual basolateral localization of the protein, or there may be a detrimental change in the interaction of AE1 with some other intracellular component (11). Further work will be required to explore these possibilities.

Two other observations also support the notion that the mechanism by which these mutations impair acid secretion is not simply related to loss of ion transport. First, were this the case, we would expect to find a wide variety of mutations in this gene among patients with dominant dRTA, rather than the preponderance of Arg-589 mutations. These might include premature termination codons, frameshift mutations, deletions, or missense mutations at many sites in *AE1*. Secondly comes consideration of mutations in *AE1* that cause the dominant red cell morphologic diseases hereditary spherocytosis and ovalocytosis. In the former, >20 *AE1* mutations have been described, including mutations that result in very early termination (codon 81) or frameshift (codon 170) (29). These mutations have not thus far been associated with classical dRTA, although a recent report identifies two patients from one family with coexistent spherocytosis and defective urine acidification (30). Two earlier reports of patients with coexistent dRTA and ovalocytosis or elliptocytosis (31, 32) did not include the demonstration of cosegregation of these traits, nor mutation analysis; fortunately, however, it was this coexistence that prompted the initial consideration of *AE1* as a candidate gene for dRTA (32).

The phenotypic similarity between the affected brothers missing the last 11 amino acids of AE1 and the subjects with Arg-589 mutations suggests that these mutations impart similar effects on AE1 function. To date, the role of the intracellular C terminus of this molecule remains to be elucidated. It is possible either that there is a significant interaction between the two domains, or again that correct membrane targeting of the molecule is affected. Future functional studies will therefore be of great interest, and illustrate the utility of rare disorders such as RTA in elucidating normal physiological function.

Review of the clinical histories in our patients reveals differences between patients with dominant and recessive disease that may prove useful in screening of future patients. Age at presentation was much younger in our recessive patients, index cases often being diagnosed in infancy. Hypokalemia was more severe, and all recessive patients exhibited growth retardation. Nephrocalcinosis was also a universal finding in this subset, even in a neonate. In contrast, the dominant patients were identified at older age, and none of the affected members of kindred RTA10 that we studied has radiological evidence of nephrocalcinosis. Even so, there is still a spectrum of clinical severity within previously reported dominant disease (33, 34); and while many of our patients are entirely asymptomatic, patient RTA8–1 was growth impaired, and patients RTA6–1, -6–2, and the propositae in kindred RTA10 presented with renal stone disease (19). We therefore suggest that clinical presentation in adolescence or adulthood, or achievement of normal stature while untreated, is strongly suggestive of dominant disease, and should motivate a search for *AE1* mutations as well as screening of first degree relatives. The absence of a family history, as illustrated by the *de novo* mutation in patient RTA8–1, does not preclude such a search.

There is also phenotypic variation among our recessive patients, which suggests that recessive dRTA is likely to be heterogeneous. For example sensorineural deafness, while cosegregating with the RTA, is present only in a subset of patients. It is notable that recessive disease without evidence of deafness, as found in many of our patients, has to date been much less commonly reported. Having excluded *AE1* as a candidate, consideration of other appropriate candidate genes will be required to elucidate further the genetic basis of these conditions, using both analysis of linkage and direct screening. As shown in Fig. 1, such candidates might include genes for subunits of the proton transporters (H^+- or H^+/K^+-ATPases) or for other elements in the metabolic pathways that mediate or regulate acid secretion in collecting duct cells.

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