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Dual Transport Properties of Anion Exchanger 1

THE SAME TRANSMEMBRANE SEGMENT IS INVOLVED IN ANION EXCHANGE AND IN A CATION LEAK[S]

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Previous results suggested that specific point mutations in human anion exchanger 1 (AE1) convert the electroneutral anion exchanger into a monovalent cation conductance. In the present study, the transport site for anion exchange and for the cation leak has been studied by cysteine scanning mutagenesis and sulfhydryl reagent chemistry. Moreover, the role of some highly conserved amino acids within members of the SLC4 family to which AE1 belongs has been assessed in AE1 transport properties. The results suggest that the same transport site within the AE1 spanning domain is involved in anion exchange or in cation transport. A functioning mechanism for this transport site is proposed according to transport properties of the different studied point mutations of AE1.

Recent work on membrane permeability has shown the ambiguity in transport mechanism between channels and transporters (for reviews, see Refs. 1–3). The membrane proteins involved in substrate movements through the lipid bilayer have been traditionally divided into three groups according to mechanistic and structure specificities: transporters, channels, and ionic pumps. However, the observations that neurotransmitter transporters can function as chloride channels (4, 5), that palytoxin-modified Na,K-ATPase behaves like a conductance (6), and that chloride channels are deregulated Cl^-/H^+ exchangers (7; for review see Ref. 8) have blurred the distinction between transporters and channels.

Our work on anion exchangers suggests that these proteins also exhibit unexpected behavior for classical transporters. Anion exchanger 1 (AE1)[2] belongs to the SLC4 gene family and has been known for half a century to be an electroneutral anion exchanger facilitating Cl^- and HCO_3^- movements through the plasma membrane. AE1 is expressed mainly in red cells and kidney α-intercalated cells. It is multifunctional in red cell physiology: it contributes to red cell shape and senescent erythrocyte recognition, and it is involved in respiration through CO_2 transport and in metabolism by regulating glycolytic enzyme activity (9). We have studied AE1 transport function in trout red cells and shown that trout AE1 has a dual transport mechanism, displaying both anion exchanger and anion conductance permeable to taurine, Na^+, and K^+ (10–12). This anion conductance is involved in trout red cell regulatory volume decrease and activated by a decrease in intracellular ionic strength (13, 14). Certain variant human red cells also show conductive activity associated with AE1 in response to some specific point mutations (15). However, in these variants human AE1 point mutations induce a cation conductance nonselective for Na^+ and K^+ (16). Depending on the mutations, we have proposed that the exchanger is either converted to a nonselective cation conductance or it can behave simultaneously as an anion exchanger and as a nonselective cation conductance (17–19). The specific point mutations involved in changes of the transport mechanism are associated with human pathologies such as distal renal tubular acidosis or hereditary hemolytic anemia (20–23).

To characterize further the cation leak induced by AE1 point mutations, the present work intends to confirm that cations move through mutated AE1 and to localize the cation pathway within the AE1 spanning domain. The question arises as to whether anions and cations move through the same pathway in wild-type (WT) and mutated proteins or whether two different pathways may coexist: one for anion exchange and another one for cation conductance, opened by specific point mutations in a different part of AE1 spanning domain.

Numerous biochemical and biophysical studies have provided information on the AE1 spanning domain and its transport site. The putative secondary structure proposes 12–14 α-helices spanning the lipid bilayer (24, 25). Dr. Joe Casey's group has shown that putative TM8, 9, 12, and 13 contain important amino acids for anion exchange (26, 27) (Fig. 1). Moreover, the extracellular loop between TM7 and TM8 contains a selectivity filter for anions as does the top of helices 12 and 13. Using the cysteine scanning mutagenesis and sulfhydryl-specific chemical labeling techniques, Dr. Casey's group proposes that TM8 forms part of the transmembrane pore lining (28). Similarly, the α-helix in NBCe1 (sodium/bicarbonate cotransporter) corresponding to AE1 putative TM8 was also shown to participate to the anion translocation site of this other member of SLC4 family (29).

In the present study, to assess whether the same transport pathway is involved in anion exchange of WT AE1 and in cation conductance of the point-mutated AE1, the cysteine scanning mutagenesis and sulfhydryl-specific chemical labeling tech-

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: AE1, anion exchanger 1; MBS, modified Barth's saline; MTSEA, 2-aminoethyl methane thiosulfonate hydrobromide; MTSET, 2(trimethylammonium)ethyl methane thiosulfonate bromide; NBC, sodium/bicarbonate cotransporter; NI, noninjected; PCMBS, parachloromercuribenzene sulfonate; TM, transmembrane.

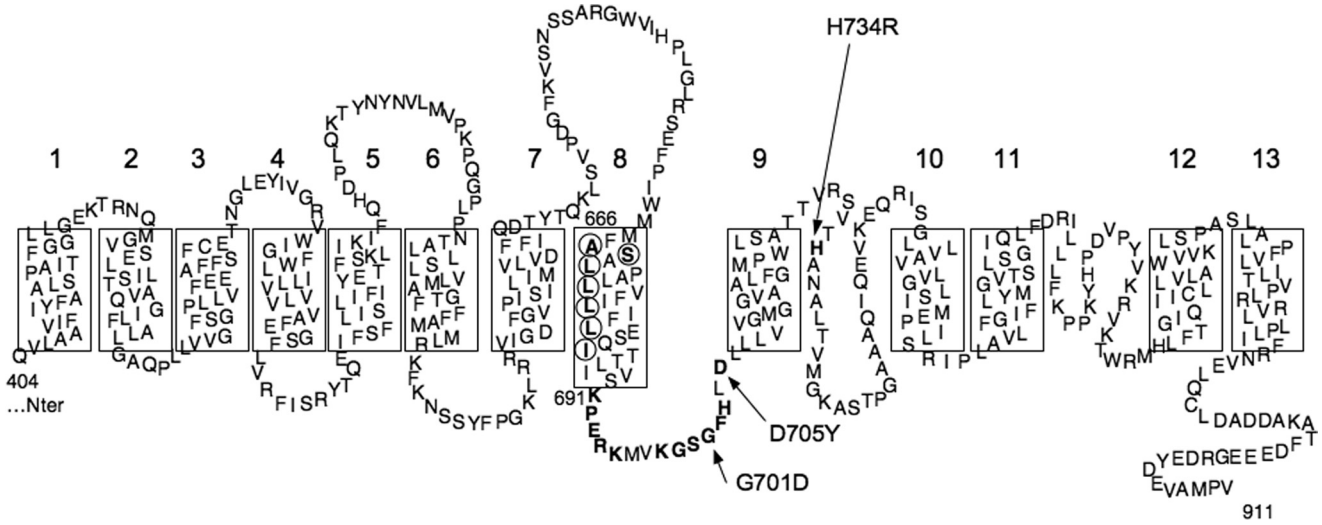

FIGURE 1. **Putative topology of AE1-spanning domain according to Zhu *et al.* (26).** The point mutations made in TM8 and in the intracellular loop between TM8 and TM9 are highlighted by *bold characters*. *Arrows* point out amino acid substitutions responsible for human pathologies and used in this study.

niques were used on a cation-conductive AE1 (point mutation H734R) (16). The involvement of critical amino acids for anion exchange activity in TM8 was assessed on cation transport. Moreover, we focused on the intracellular region connecting putative TM8 to TM9 which contains a highly conserved sequence among all SLC4 members as well as many positive and negative charges. The role of these charges and conserved amino acids was assessed by specific point mutations.

Our data suggest that the same ion pathway through the AE1 spanning domain is involved in either anion exchange or cation leak. Moreover, the connecting region between TM8 and TM9 seems to play a key role in ion selectivity of this pathway. Two key residues within this region are Lys-695 and Lys-698, which are required for the protein to operate as an anion exchanger. Substitution of either of these two lysines strongly reduces anion exchange and induces a Na^+ and K^+ transport.

These data greatly support the hypothesis that point mutated AE1 itself can transport cations. Moreover, this implies that only small changes are needed in the primary structure of AE1 to convert the transport mechanism of AE1 from an obligate exchanger to a channel.

EXPERIMENTAL PROCEDURES

Construction of hAE1 cDNA with Point Mutations: P692L, K691Q, E693Q, R694/G/Q/D, K695L, K698C/G/D/Q, G699C, S700C, G701C/K, F702C, H703C, and D705C/G/K—Site-directed mutagenesis was performed on WT human erythroid AE1 cloned in pSP65 using the QuikChange site-directed mutagenesis kit (Stratagene) with primers covering 15 bases upstream and downstream the mutation (Eurogentec, Seraing, Belgium). One positive clone for each mutation was sequenced before further use (Beckman Cogenics, Takeley, UK). The plasmid with D705Y mutation has been described previously (16). The cysteine substitutions in putative TM8 (A666C, S667C, L669C, L673C, L677C, L680C, and I684C) were performed on WT AE1 and on H734R point-mutated AE1 with the same protocol.

Oocyte Injection—Isolation and cRNA injection of oocytes were performed as described previously (12). Briefly, HindIII-linearized pSP65hAE1WT and mutant plasmids were transcribed by SP6 RNA polymerase (Ambion transcription kit). Female *Xenopus laevis* were anesthetized with MS222 according to the procedure recommended by our ethics committee.

Stage V–VI oocytes were injected with 10 ng of AE1 (WT and mutated) cRNA. Injected oocytes were maintained at 18 °C in MBS for 3 or 4 days before running the experiments. Modified Barth's saline (MBS composition in 85 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, 4.5 mM NaOH, pH 7.4, supplemented with 10 units/ml penicillin and 10 μg/ml streptomycin). For Na^+ and K^+ content measurements, oocytes were incubated in MBS with 0.5 mM ouabain and 5 μM bumetanide. Cl^-/HCO_3^- exchange activity, Na^+ and K^+ contents, Li^+ uptake, and pH_i measurements were determined simultaneously on the same batch of oocytes 3 or 4 days after injection.

Oocyte Na^+ and K^+ Content Measurements—As published previously (12), oocytes incubated in MBS with ouabain and bumetanide were quickly washed two times in 7.5 ml of MilliQ water (Millipore) and dried on aluminum foil overnight at 80 °C after removing excess extracellular fluid. Dried oocytes were weighed to determine dry cell solids. Intracellular ions were extracted by suspending dried oocytes in 4 ml of MilliQ water overnight at 4 °C. Measurements of sodium and potassium were done with flame spectrophotometer (Eppendorf). For each experiment, a triplicate was made for each condition, and results were expressed as micromoles/gram of dry cell solids.

Cl^-/HCO_3^- Exchange Activity Measurements—Oocyte intracellular pH was measured using selective microelectrodes as published previously (16). The ability of WT and mutant hAE1 to regulate intracellular pH was assessed by measuring intracellular pH of oocytes acidified by incubation in the following medium: 63.4 mM NaCl, 1 mM KCl, 24 mM HCO_3^-, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 5 mM HEPES/NaOH, pH 7.35, 5% CO_2, 95% O_2 and then bathed with a MBS without Cl (63.4 mM sodium gluconate, 1 mM potassium gluconate, 24 mM HCO_3^-, 0.82 mM $MgSO_4$, 0.74 mM $Ca(NO_3)_2$, 5 mM HEPES/NaOH, pH 7.35, 5% CO_2, 95% O_2). Results were given

in ΔpH_i/min ± S.E. ΔpH_i was measured when acidified oocytes were exposed to Cl-free medium. It corresponds to the initial slope of the alkalinization.

Thiolalkylations—Thiolalkylation of cysteines was performed by oocyte incubation for 15 min at room temperature in MBS with 0.2 mM parachloromercuribenzene sulfonate (PCMBS) or 5 mM 2-aminoethyl methane thiosulfonate hydrobromide (MTSEA) extemporaneously prepared from stock solutions 1 M in dimethyl sulfoxide stored at −20 °C. For 2(trimethylammonium)ethyl methane thiosulfonate bromide (MTSET), it was weighted and solubilized in MBS at 5 mM just before use. After 15 min, oocytes were washed in MBS and assessed directly for lithium uptake experiments or pH_i measurements.

Lithium Uptake—Lithium was used as a substitute for Na^+ to measure oocyte cation permeability. To prevent cation movements through the Na,K-ATPase or the Na,K 2Cl cotransporter, lithium uptake measurements were done in the presence of 5 μM bumetanide and 0.5 mM ouabain in $LiNO_3$ MBS (85 mM $LiNO_3$, 1 mM KNO_3, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, 4.5 mM NaOH, pH 7.4). In each experiment, seven oocytes/condition were incubated for 2 h in $LiNO_3$ MBS at 19 °C. As described previously (16), lithium in oocytes was quantified by atomic absorption spectrometry with a PerkinElmer Life Sciences AAS3110.

AE1 Immunodetection on Oocyte Plasma Membrane—To detect AE1 addressed to oocyte plasma membrane, surface proteins of oocytes expressing WT or mutated AE1 were labeled with sulfo-NHS-SS-Biotin (Pierce) for 15 min in MBS, pH 8, at 4 °C. The reaction was then quenched with buffer (192 mM glycine, 25 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$), and oocytes were washed in MBS, pH 8. Oocytes were then lysed mechanically in homogenization buffer (20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 0.5 mM EDTA, 0.5 mM protease inhibitor Pefabloc (Roche Applied Science)), and oocyte membranes were prepared as published previously (30). The oocyte membranes were then solubilized in radioimmune precipitation assay buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.5 mM Pefabloc for 30 min at 4 °C). Biotin-labeled proteins in the solubilized membranes were isolated by immobilization on avidin resin (Pierce) for 30 min at 4 °C. After washing in radioimmune precipitation assay buffer, biotin-labeled proteins were eluted in SDS-PAGE loading buffer and analyzed on SDS-PAGE, transferred to a PVDF membrane (Millipore), and probed with primary antibodies as indicated in the figure legends. Secondary antibodies were anti-rabbit or anti-mouse horseradish peroxidase-coupled (Sigma) and detected by chemiluminescent reaction with Immobilon Western reagent (Millipore) and a Fusion FX7 (Vilber-Lourmat, France). To compare AE1 expression levels in different samples, the cell membrane marker β1 Na,K-ATPase was used. The intensity of each AE1 band relative to the total membrane fraction (β1 Na,K-ATPase signal) was quantified in each lane with National Institutes of Health ImageJ.

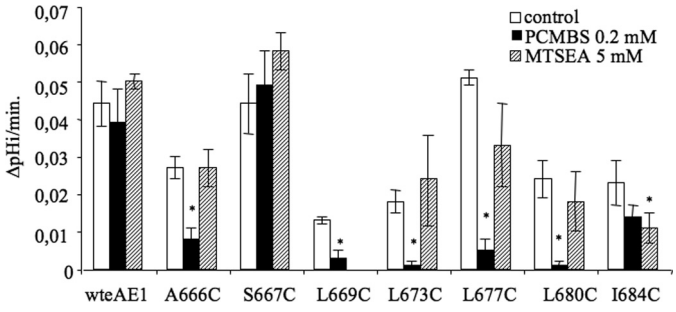

FIGURE 2. **Cl^-/HCO_3^- exchange activity of WT AE1 and cysteine mutants.** Oocytes were acidified by incubation in medium with 24 mM HCO_3^-/5% CO_2; at equilibrium extracellular chloride was substituted by gluconate, which should induce an alkalinization in presence of functional Cl^-/HCO_3^- exchange. The slope of the initial alkalinization as a function of time was calculated from intracellular pH recording of oocytes expressing WT or different cysteine mutants. The oocytes were in control condition (no treatment by alkylating agents, *white bars*) or treated by 0.2 mM PCMBS (*black bars*) or 5 mM MTSEA (*striped bars*). Thiol reagent treatments were done in MBS for 15 min, and then oocytes were washed in MBS before pH_i measurements. Data presented are means ± S.E. (*error bars*) of 10 (wtAE1), 17 (A666C), 7 (S667C), 8 (L669C), 15 (L673C), 8 (L677C), 9 (L680C), and 11 (I684C) oocytes coming from two or three different batches. In supplemental Fig. 1 are presented traces of pH_i recordings for oocytes expressing cysteine mutants with or without thiol reagents. For each mutant, Student's *t* test was performed to compare mean values in control condition or in presence of MTSEA or PCMBS. *, $p < 0.05$.

RESULTS

Cysteine Scanning Mutagenesis in Putative TM8—The cysteine scanning mutagenesis method relies on accessibility of substituted cysteine. Each residue in the membrane-embedded segments of the transport protein is mutated one at a time to Cys, the mutant is expressed in heterologous cells, and the susceptibility of these substituted cysteines to reaction with small/large, charged/neutral, sulfhydryl-specific reagents is determined. If application of the reagent results in an irreversible alteration of the function of the transporter, it is inferred that the substituted Cys reacted and, therefore, was exposed in the water-filled lumen of the transporter (31).

Introduction of cysteine in place of Ala-666, Ser-667, Leu-669, Leu-673, Leu-677, Leu-680, and Ile-684 was done in WT AE1 (containing two endogenous cysteines in the spanning domain) and in a cation conductive AE1 with the point mutation H734R (16). It was not possible to work with the Cys-less AE1 (kind gift from Dr. Joe Casey) because this was not expressed in the oocyte plasma membrane at a level allowing detection of Cl^-/HCO_3^- exchange activity. Thus, thiolalkylations were done in parallel, on control WT AE1 and on AE1 with specifically introduced cysteines. Thioalkylating agents with different charges and size were used: in order from the larger to the smaller: PCMBS (anionic), MTSET (cationic), or MTSEA (cationic and partially neutral 10/1 at pH 7.5, the neutral form is membrane-permeant and can react with intracellular accessible cysteines). Reaction of WT AE1 with thiolalkylating agents (PCMBS or MTSEA) did not change Cl^-/HCO_3^- exchange activity, suggesting that the endogenous cysteines were not accessible to the reagents or located in a position not involved in anion exchange activity (Fig. 2). Positions Ala-666, Ser-667, Leu-669, Leu-673, Leu-677, Leu-680, and Ile-684 in putative TM8 have been proposed to line the anion translocation site in a Cys-less AE1, similar residues line the translocation site in NBCe (28, 29). To confirm these data in the *Xenopus*

oocyte expression system, the Cl^-/HCO_3^- exchange activity was measured in oocytes expressing AE1 with the introduced cysteines in TM8 in the presence or absence of 5 mM MTSEA or 0.2 mM PCMBS (Fig. 2). Confirming the previous study in HEK293 cells (28), PCMBS was able to inhibit anion exchange in oocytes expressing A666C, L669C, L673C, L677C, and I680C AE1 mutants. No inhibition was observed with oocytes expressing I684C and S667C (in contrast to the previous study) (28).

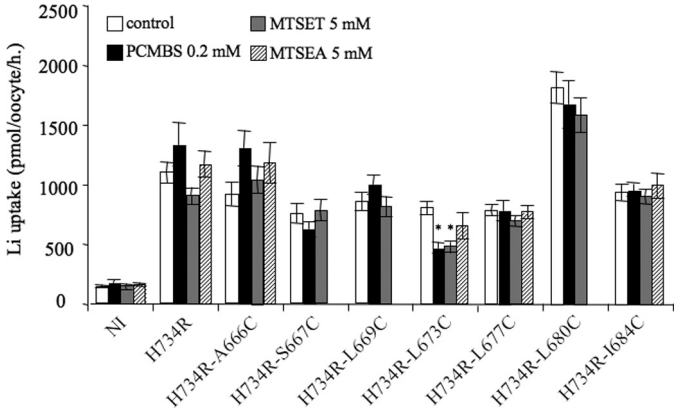

FIGURE 3. **Li^+ uptake in oocytes expressing WT AE1, H734R single mutant, or H734R mutation with introduced cysteine in TM8.** Just before the uptake experiment, oocytes were incubated for 15 min in MBS with different thiolalkylating agents (PCMBS, *black bars*; MTSET, *gray bars*; MTSEA, *striped bars*). The uptake was done in $LiNO_3$ medium with ouabain and bumetanide as mentioned under "Experimental Procedures." Data presented are means ± S.E. (*error bars*) of 10–46 oocytes coming from 2–9 different experiments. *, $p < 0.05$ for each mutant a *t* test comparing control condition (without any reagent) with MTSET, PCMBS, or MTSEA conditions.

MTSEA was able to block Cl^-/HCO_3^- exchange significantly only in oocytes expressing the I684C mutant whereas Tang *et al.* (28) have also observed anion exchange inhibition (around 25%) by MTSEA on positions 673 and 677 in HEK293 cells.

The cation transport activity of AE1 mutant H734R with cysteines in TM8 was also assessed in presence or absence of three alkylating agents: PCMBS, MTSEA, and MTSET as shown in Fig. 3. The H734R mutant cannot exchange anions (Cl^- and HCO_3^-), and it was suggested that the mutation converts the electroneutral anion exchanger into a nonselective Na^+,K^+ conductance (16). None of the cysteine mutants on H734R AE1 was able to exchange anions (Cl^- and HCO_3^-). Moreover, introduction of cysteines along TM8 did not change the cation leak, and all the double mutants were cation-leaky (Fig. 3). Reaction with PCMBS, MTSEA, or MTSET did not significantly modify Li^+ uptake in any oocytes expressing the H734R mutant with A666C, S667C, L669C, L677C, L680C, or I684C substitutions. However, for oocytes expressing H734R/L673C, Li^+ uptake was significantly decreased by PCMBS (−43 ± 6%) and MTSET (−42 ± 6%).

Involvement of the Intracellular Region Connecting Putative TM8 and TM9 in Transport Properties—The alignment of SLC4 sequences from different species in the intracellular region between putative TM8 and TM9 of human AE1 shows a highly conserved sequence K*G*X*G*X*H*X*D among these different proteins, all of which are involved in bicarbonate transport (electroneutral anion exchangers AE1, AE2, AE3, and sodium bicarbonate cotransporters NBC; Fig. 4). In this motif, substi-

Species/transporter	Sequence identification	Amino acids	position
rabbit NBC	O97915	NRKEHKLK**KGAGYHLD**	763
rat NBC	O35422	NRKEHKLK**KGAGYHLD**	763
Human NBC	O15153	NRKEHKLK**KGAGYHLD**	763
human NBC	O60350	NRKEHKLK**KGAGYHLD**	725
human NBC	O95233	NRKEHKLK**KGCGYHLD**	809
C. elegans	Q20642	NRKENKLK**KGCGYHLD**	804
mouse NBC	O88343	NRKEHKLQ**KGAGYHLD**	807
salamender NBCe	O13134	NRKEHKLK**KGAGYHLD**	763
chicken AE2	Q90710	SKKERML**QKGSGFHLD**	999
human AE2	B3A2_HUMAN	SKKERML**QKGSGFHLD**	1019
rabbit AE2	B3A2_RABIT	SKKERML**QKGSGFHLD**	1016
mouse AE2	B3A2_MOUSE	SKKERML**QKGSGFHLD**	1016
green puffer AE2	Q4RMY1	SKKERML**VKGSGFHLD**	1063
zebra fish AE2	Q3KVL6	SKKERML**VKGSGFHLD**	1007
skate AE2	Q7T1P5	SKKERML**TKGSGFHLD**	996
guinea pig AE2	Q9Z0S8	SKKERML**QKGSGFHLD**	1017
rat AE2	B3A2_RAT	SKKERML**QKGSGFHLD**	1013
mouse AE3	B3A3_MOUSE	SQKARRL**LKGSGFHLD**	1007
human AE3	B3A3_HUMAN	SQKARRL**LKGSGFHLD**	1012
rat AE3	B3A3_RAT	SQKARRL**LKGSGFHLD**	1007
rabbit AE3	O18917	SQKARRL**LKGSGFHLD**	1013
Green puffer AE3	Q4SK68	SKKERRL**VKGSGFHLD**	554
skate AE3	Q7T1P4	SKKERHL**VKGSGFHLD**	998
human AE1	B3AT_HUMAN	SKPERKM**VKGSGFHLD**	690
mouse AE1	B3AT_MOUSE	SKPERKM**IKGSGFHLD**	708
rat AE1	B3AT_RAT	SKPERKM**IKGSGFHLD**	706
trout AE1	B3AT_ONCMY	SKPERKM**VKGSGFHLD**	703
chicken AE1	B3AT_CHICK	SKPERKL**VKGSGFHLD**	701
zebra fish AE1	D1FTM8	SKPERKM**VKGSGFHMD**	632
skate AE1	Q7P1P6	SKPDRKM**VKGSGFHLD**	677
eelpout AE1	B2CX97	SKPERKM**VKGSGFHFD**	632
Drosophila AE	Q9VT48	DKPDRGL**KKGSGLHWD**	
Anopheles AE	Q7PP75	DKPERGL**KKGSGLHMD**	
Conserved sequence in SLC4		KG G H D	
Conserved charges in AE1		+ -++ + -	

FIGURE 4. **Alignment of conserved amino acids in the putative region between TM8 and TM9 (16 amino acids) in SLC4 family members.** Identical amino acids are in *bold characters*, and conserved residues are in *gray*.

tution of the second Gly or the Asp in human AE1 (G701D and D705Y) is associated with a cation leak when expressed in *Xenopus* oocytes or in red cells as published previously (15). We substituted each of Lys-698, Gly-699, Ser-700, Gly-701, Phe-702, and His-703 with cysteine to assess the role of each amino acid of this sequence in AE1 transport properties. Moreover, the alignment shows an equivalent number of 4 positive charges at the beginning of the putative intracellular loop (KPERK*XX*K in human AE1) and a negative charge at the end of the loop (Asp-705 in human AE1). There is also a conserved negative charge (Glu-693 in human AE1) between the four positive charges (except in mammalian AE3). To assess the involvement of these charges in human erythroid AE1 transport features, the following substitutions have been done: K691Q, E693Q, R694G/Q/D, K695L, K698G/Q/D, and D705C/G/K. The conserved Pro in AE1 sequences was also substituted (P692L). All of the mutated AE1 have been expressed in *Xenopus* oocytes, and their expression in the oocyte plasma membrane and their transport features (chloride/bicarbonate exchange and cation transport) have been assessed.

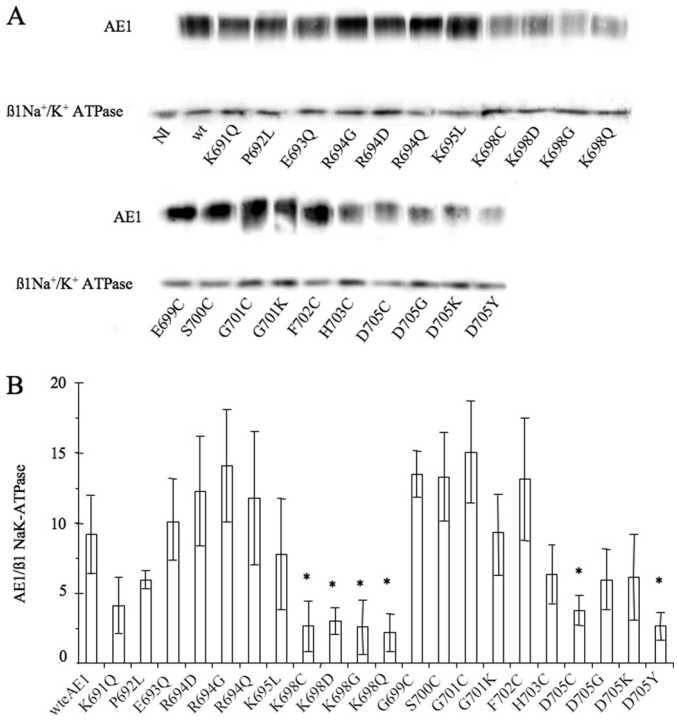

FIGURE 5. **Immunodetection of WT and mutant AE1.** *A*, Western blots for immunodetection of AE1 in biotin-labeled oocyte plasma membrane samples (*upper row*). Primary antibody was an anti-N-terminal domain of AE1 1/10,000 (CDB3, kind gift from Dr. P. Low). *Lower row*, immunodetection of β1 Na,K-ATPase (43 kDa) in the same samples (primary antibody clone M17-P5-F11, 1/250; Sigma). The figure combines three Western blots, showing all of the different AE1 constructs. The Western blots were chosen according to their similarity with the quantification data shown in Fig. 5*B*. *B*, quantification of AE1 level in plasma membrane normalized to β1 Na,K-ATPase signal. After corresponding background subtraction, pixels corresponding to AE1 signal were divided by pixels corresponding to β1 Na,K-ATPase signal in the same biotin-labeled sample. Each *bar* is a mean ± S.E. (*error bars*) of at least three different Western blots corresponding to different batches of injected oocytes. Student's *t* test was performed comparing WT AE1 data with mutant AE1 data (*, $p < 0.07$).

All of the studied mutants were expressed in the oocyte plasma membrane as shown in Fig. 5. The *large error bars* do not allow us to infer significant differences regarding plasma membrane expression between the mutants. This variability is mainly due to great differences between faint endogenous β1 Na^+,K^+-ATPase expression and overexpression of injected AE1. However, the signal associated with K698C/D/G/Q, D705C/Y mutants, was significantly lower than for WT AE1. This could reflect a lower expression level in the oocyte plasma membrane. Another explanation could be that the mutations impair biotin accessibility by remodeling extracellular loops, thus leading to lower level of biotin labeling.

The Cl^-/HCO_3^- exchange activity of the different mutants was assessed as previously described (Fig. 6, *white bars*). After an acid load in CO_2/HCO_3^--buffered medium, the intracellular pH recovery in Cl-free medium as a function of time was plotted for the WT AE1 and the different mutants. It was observed that AE1 with the substitutions K691Q, P692L, E693Q, R694D/G/Q, G699C, S700C, G701C, F702C, H703C, and D705C/G were functional in terms of anion exchange. This suggests that these mutants were folded correctly to operate anion exchange. Conversely, all of the substitutions on Lys-698 strongly reduced the anion exchange capacity of the protein with a dramatic effect by Asp substitution. Similarly, the K695L mutation abolished anion exchange activity. The effect of substitutions on position Gly-701 depended on the introduced amino acid: G701K could not exchange Cl^- and HCO_3^- whereas G701C could. The same thing was observed with the Asp-705 mutants: substitution by Cys or Gly was functional for anion exchange,

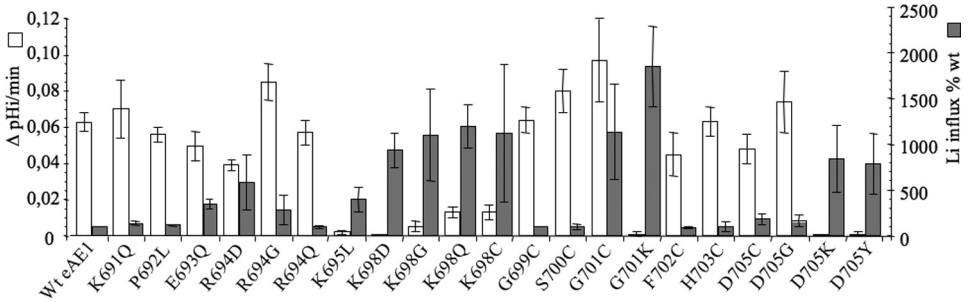

FIGURE 6. **Cl^-/HCO_3^- exchange activity and Li^+ uptake in oocytes expressing WT or mutated AE1.** *White bars*, Cl^-/HCO_3^- exchange activity. The slope of initial alkalinization in gluconate-CO_2/HCO_3^- medium was plotted as a function of time. Data are means ± S.E. (*error bars*) of 4–42 oocytes (depending on mutants) coming from at least two experiments. *Gray bars*, Li^+ uptake. Data are means ± S.E. of at least three experiments (each of them with 7 oocytes/condition). To plot on a single figure data from different experiments with varying base line, in each experiment mean Li^+ uptake for mutants was expressed as a percent of control oocytes (noninjected or expressing WT AE1). Mean ± S.E. Li^+ uptake was in pmol/oocyte per h: 106 ± 99 ($n = 34$ experiments) for NI and 71 ± 64 ($n = 28$ experiments) for WT AE1. Statistical analyses of the data have been done with Student's *t* test comparing fluxes between WT and mutant-expressing oocytes.

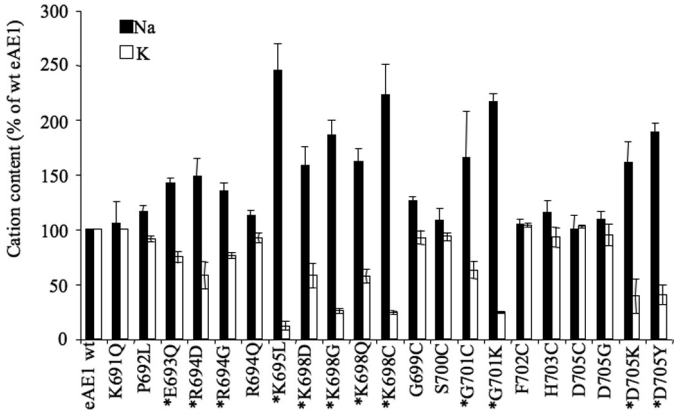

FIGURE 7. **Oocyte Na^+ and K^+ contents.** Na^+ and K^+ contents of oocytes expressing WT or mutated AE1 are shown. Data are means ± S.E. (*error bars*) of at least three experiments. For each single experiment, measurements were done on triplicate for each condition and mean Na^+ or K^+ contents were expressed in percent of control oocytes (noninjected or expressing WT AE1). Student's *t* test was performed to compare data from mutant-expressing oocytes with WT-expressing oocytes (*, $p < 0.07$).

whereas Lys or Tyr substitutions were not. This lack of anion exchange activity did not result from a defect in processing to the oocyte plasma membrane as shown in Fig. 5. However, a lower anion exchange activity could be due to a reduced number of protein at the cell surface. Because some substitutions on Lys-698 exhibit a faint but significant anion exchange activity, it remains possible that a better expression of these mutants at the plasma membrane would have increased the anion exchange activity. However, for a similar expression level, D705C exhibits a much better anion exchange activity than Lys-698 mutants, for instance. Thus, it is quite difficult to correlate the expression level to the intensity of the exchange activity but the absence of anion exchange activity is not due to the absence of AE1.

The cation permeability of oocytes expressing the different mutants was then assessed by measuring Li^+ uptake (Fig. 6, *gray bars*). A significant increase in Li^+ permeability is observed for mutants E693Q, R694/D/G, K695L, K698D/G/Q/C, G701C/K, D705K/Y ($p < 0.05$). All of the studied mutants were able to induce anion exchange activity and/or Li^+ uptake. However, there is no correlation between the level of AE1 expression in oocyte plasma membrane and the intensity of anion exchange activity or Li^+ uptake. Similar membrane expression levels give different amounts of anion exchange activity: G701C *versus* G701K mutants, for instance. The reduced accumulation of Lys-698 mutants in plasma membrane is associated with low anion exchange activity and with high Li^+ permeability of oocytes expressing these mutants. In contrast, E693Q, R694D, R694G, and G701C exhibit a lower cation leak and higher anion exchange whereas they are more abundant in oocyte plasma membrane than Lys-698 mutants.

The increased Li^+ permeability is correlated with changes in oocyte cation content as illustrated in Fig. 7. Fig. 7 illustrates the cation contents of oocytes expressing the different AE1 mutants. To plot on a single figure the data coming from different experiments with varying base lines, it was decided to express the data as percents of control. In each experiment, 100% was assigned to the control, *i.e.* noninjected oocytes or WT AE1-expressing oocytes, and values for oocytes expressing mutated AE1 were calculated compared with these controls. This allowed us to normalize the data between the different experiments, and the data presented are means ± S.E. The statistical significance of Na^+ and K^+ changes was determined on each individual experiment by a *t* test comparing mean values of Na^+ or K^+ contents between mutant and WT expressing oocytes. The mean values for Na^+ and K^+ contents were not statistically different between NI oocytes and oocytes expressing WT AE1 (in μmol/g of dry weight, Na^+: 48 ± 11 (NI, n = 31), 52 ± 13 (WT AE1, n = 25); K^+: 86 ± 17 (NI, n = 31), 75 ± 18 (WT AE1, n = 25). From these data, mutated AE1 with the following amino acid substitutions E693Q, R694D/G, K695L, K698D/G/Q/C, G701C/K, as well as D705K/Y exhibit a significant cation leak ($p < 0.05$). In contrast, substitutions K691Q, P692L, G699C, S700C, F702C, H703C, and D705C/G did not induce any significant Na^+ and K^+ change.

Thus, along the intracellular loop between putative TM8 and TM9, positions Glu-693, Arg-694, Lys-695, Lys-698, Gly-701, and Asp-705 seems to play an important role in the transport features of the protein, either inducing a cation permeability and abolishing or dramatically decreasing anion exchange (K698D/G/C/Q, G701K, and D705K/Y mutants) or inducing a cation permeability in a still functional anion exchanger (E693Q, R694D/G, G701C). In contrast, positions Lys-691, Pro-692, and Gly-699, Ser-700, Phe-702, and His-703 in the conserved consensus site could be substituted without any significant change in AE1 transport properties: anion exchange was maintained, and no cation permeability appeared. Comparison of Cl^-/HCO_3^- exchange and Li^+ uptake shows that a significant increase in Li^+ uptake is associated with a decrease in Cl^-/HCO_3^- exchange activity (Fig. 6). Three noticeable exceptions to this rule are mutants E693Q, R694D, and G701C, which keep a high anion exchange activity and induce a Li^+ permeability compared with WT AE1.

DISCUSSION

Involvement of AE1 TM8 in Anion Exchange and Cation Leak—A model for the anion translocation pore in AE1 has been proposed by Tang *et al.* (28). According to their observations, a pore for anion exchange through the AE1 membrane-spanning domain is widely opened on the extracellular side and involves TM8. At the intracellular part of putative TM8, residue Glu-681 was proposed to interact with transported anions, to be accessible from either side of the membrane, and to reside at the transmembrane permeability barrier (32). Tang *et al.* (28) identified residues lining the transmembrane translocation pore above and below Glu-681 and showed that this transport site forms an outward facing funnel that narrows to a permeability barrier at Glu-681. Our data of cysteine mutants in TM8 expressed in *Xenopus* oocytes generally confirmed the observations of Tangs *et al.* (28) in HEK293 cells. We showed that PCMBS blocked anion exchange when fixed in positions 666, 669, 673, 677, and 680 whereas MTSEA, which is smaller, failed to do so. This may be because these amino acids were not accessible to MTSEA. Alternatively, it may be that at pH 7.5 MTSEA is mainly positive and could not easily reach these positions in an anion-selective transporter. However, Tang *et al.* (28) were

able to observe approximately 25% inhibition of anion exchange by MTSEA on positions Cys-673 and Cys-677. Moreover, the S667C mutant was slightly inhibited by PCMBS (18%) in HEK293 cells whereas it is not sensitive in *Xenopus* oocytes. These differences in results may be because of the slightly different shape or size adopted by the mutant protein in the different plasma membrane environments of oocytes and HEK cells or because the Tang *et al.* (28) study used a cysteine-less protein. However, our data strongly suggest that in *Xenopus* oocytes as in HEK cells, AE1 TM8 forms part of the anion translocation pore.

In the cation leaky AE1 (mutant H734R), which is unable to exchange Cl^- and HCO_3^-, PCMBS and MTSET were able to block lithium uptake when fixed on position Cys-673. This strongly suggests that the anion exchange pathway involving TM8 is also involved in the cation leak. Moreover, the shape of the cation pathway is also probably similar to a funnel as for the anion exchange pathway. The inhibition of cation transport by PCMBS or MTSET on C673R/H734R mutant is lower than the inhibition of anion exchange by these reagents on the single Cys-673 mutant. This suggests that the transport pathway in the C673R/H734R, cation-leaky mutant, is still large enough for a few cations to move despite the presence of PCMBS or MTSET. In agreement with this interpretation, the smaller reagent MTSEA failed to block cation movement in L673C/H734R mutant. PCMBS does not block cation movement on cysteine 677 and 680, suggesting that these positions are not accessible in the cation-leaky conformation. A constriction in AE1 transport pathway around position Leu-673 could be induced by H734R mutation, thus changing AE1 transport selectivity.

Role of Intracellular Region between TM8 and TM9 in Ion Selectivity and Transport Mechanism of AE1—Until now, the cation leak and impaired anion exchange activity of mutated AE1 have been linked to human hereditary pathologies and associated with cation leaky red cells (except for mutations involved in distal renal tubular acidosis). With the present work, we extend the list of point mutations able to induce a cation leak and to impair anion exchange function, to positions not known to be associated with pathologies (Glu-693, Arg-694, Lys-695, and Lys-698). These residues are located in the putative intracellular region between TM8 and TM9. Our data show that this domain contains highly important residues for anion exchange activity. It has been suggested that this intracellular domain starts with a helical structure at basement of helix 8 below the lipid bilayer (26, 28). Thus, it is proposed that changes in amino acid residues in this domain could alter TM8 spatial organization and consequently impair AE1 transport features. This intracellular domain contains a conserved sequence among SLC4 members (KG-G-H-D). Interestingly, substitution by cysteine of Gly-699 and His-702 in this sequence did not affect significantly anion exchanger function. However, substitutions on positions Lys-698, Gly-701, and Asp-705 greatly affect AE1 function. Thus, this conserved sequence must play a critical role in SLC4 transport features. There are many charged amino acids in the whole intracellular region between TM8 and TM9 which could be important for AE1 functioning. Indeed, substitutions on Glu-693, Arg-694, Lys-695 as well as Lys-698 and Asp-705 discussed above, modified transport characteristics of AE1 whereas substitution on Lys-691 failed to do so, suggesting that this charge is neither involved in anion exchange function nor in cation leak.

The functional consequences of amino acid substitutions on positions Glu-693, Arg-694, Lys-695 and Lys-698, Asp-705, and Gly-701 let us propose a possible function for these residues in AE1 transport features. Substitutions on Glu-693 and Arg-694 do not impair anion exchange activity but induce a small cation leak, depending on the size of the amino acid substitution: the R694Q mutant is similar to WT AE1, its substitution by a negative charge (Asp) induces a cation leak as does the substitution by a small, non polar amino acid (Gly). Thus, Glu-693 and Arg-694 are not involved as charges in anion exchange conformation of the protein but could play a steric role to prevent cation leak. In contrast, it is not possible to substitute Lys-695 or Lys-698 without dramatically impairing the anion exchange function and inducing an important cation leak. An interpretation of this observation could be that these two positive charges form a selectivity filter for intracellular anions and are required to observe anion exchange: as soon as one of them is substituted, exchange is reduced, and cation transport appears.

Position Asp-705 is similar to Arg-694: its substitution by Cys or Gly neither altered anion exchange nor induced cation leak; however, its substitution by Lys or Tyr induced a cation leak and abolished anion exchange. Thus, it could be proposed that the Asp-705 charge is also not critical for anion exchange to operate; however, this position could favor cation transport depending on the size or charge of the amino acid substitution.

Finally, position Gly-701 is critical for AE1 transport features but also for its recognition by chaperones involved in plasma membrane trafficking: its substitution by Asp impairs trafficking to plasma membrane as shown by Yenchitsomanus *et al.* (33) and induces a cation leak in a still functional anion exchanger (18). The present data show that its substitution by Lys converts the exchanger into a cation leak whereas its substitution by Cys induces a cation leak in a still-functioning anion exchanger. Other point mutations in AE1 were shown to induce a cation leak in still-functional anion exchangers; however coexpression with AE1 chaperone glycophorin A was required to obtain significant expression to plasma membrane (17–19). This suggests that these point mutations have a more dramatic effect on AE1 conformation than that required to cause a cation leak. With the G701C mutant, there was no impaired trafficking, and the protein conducted the two transport activities, confirming that they are not mutually exclusive. Thus, if the same transport domain in AE1 is involved in both anion exchange and the cation leak, the two transport processes could function independently: the anion exchange could operate with or without the cation leak and *vice versa*.

To conclude, a schematic model for the AE1 transport site can be proposed, containing an outwardly opened funnel through lipid bilayer with a barrier for anion exchange involving Glu-681 (Fig. 8) and an intracellular selectivity filter with two positive charges repelling cations (Lys-695 and Lys-698). Modification of these charges enables cation access to the transport site which is cation leaky.

Present and already published data show that the anion exchange mechanism can function simultaneously with the

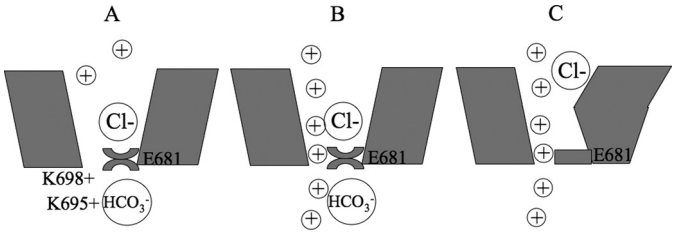

FIGURE 8. **Proposed schematic view of AE1 transport conformations.** *A*, anion exchange conformation. The two lysines in extracellular loop between TM8 and TM9 prevent cation leak. *B*, anion exchange with cation leak conformation. The two lysines moved away and exposed a pore for cation connecting both sides of the membrane; the anion exchange site is not impaired. *C*, cation leak conformation. Positive charges Lys-695 and Lys-698 moved away in cation leaky conformation, and anion exchange activity is turned down by funnel deformation, for instance.

cation leak, suggesting that the pathway itself does not discriminate charges. The Cl^-/HCO_3^- exchange should take place around Glu-681 while cations move independently of this barrier in a narrow channel along the anion exchange site. This allows us to suggest a transport mechanism between channel and transporter: conformational changes allowing anion exchange with outward and inward open forms (ping-pong model) (35) should be limited in amplitude, leaving open a channel permeable to cations. As observed, this anion exchange mechanism can easily be broken and converted into the cation leak. Thus, AE1 transport site exhibits at least three functional states: anion exchange (Fig. 8*A*), anion exchange and cation leak (Fig. 8*B*), and cation leak (Fig. 8*C*).

To account for the three transport states we would hypothesize that there must be changes in Lys-695 and Lys-698, responsible for transport selectivity, and also in the anion exchange mechanism. A single point mutation could simultaneously induce both these two changes or just the first one. Crystallographic studies on AE1 may one day confirm this picture of AE1 transport site functioning. However, it should be noted that the recent publication of the reconstruction of an outward open human erythrocyte AE1 revealed a hole through the AE1 membrane-spanning domain, connecting intra- and extracellular media (34).

The probability to open a cation leak in AE1 is increased by some specific point mutations, but it could be envisioned that other physiological or pathological agents, still unknown, could mimic the effects of the mutations on the transport conformation. This raises the question of cell membrane potential control with the existence of a potential cation leak within an electroneutral anion exchanger.

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