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Molecular mapping of the conductance activity linked to tAE1 expressed in *Xenopus* oocyte

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Abstract

It was previously shown that expressed in *Xenopus* oocyte the trout (tAE1) and the mouse (mAE1) anion exchangers behave differently: both elicit anion exchange activity but only tAE1 induces a transport of organic solutes correlated with an anion conductance.

In order to identify the structural domains involved in the induction of tAE1 channel activity, chimeras have been prepared between mouse and trout AE1. As some constructs were not expressed at the plasma membrane, skate exchanger (skAE1) was used instead of mouse exchanger to complete the structure–function analysis. The present paper shows that skAE1, highly similar to mAE1, does not induce a chloride conductance when expressed in *Xenopus* oocyte. Construct expression analysis showed that only tAE1 transmembrane domain is linked to the anion conductance. More precisely, we identified two regions composed of helices 6, 7 and 8 and putative helices 12 and 13 which are required for this function.

Keywords: Chloride/bicarbonate exchange; Band 3; Chloride channel; Red blood cell; *Xenopus* oocyte

1. Introduction

Anion exchanger 1 (AE1), also called Band 3, is the most abundant integral protein present in erythrocyte membrane (1.2×10^6 copies per cell) [1]. The primary function of AE1 consists in the electroneutral exchange of chloride and bicarbonate across plasma membrane. To date, three different genes named AE1, AE2 and AE3 are known, each of them coding for different polypeptide products depending on splicing and transcription initiation site (for review, see Refs. [2,3]). All the AE polypeptides can be divided in two main domains of about the same size: an N-terminal cytoplasmic domain and a membrane domain spanning the lipid bilayer 12–14 times with a short C-terminal end in the cytoplasm. Data obtained from topological studies make difficult to establish the number of transmembrane domains between helix 10 and the carboxy-terminal end [4]. The isoforms differ mainly in their N-terminal cytoplasmic domain, the membrane spanning domain showing great similarities and being responsible for the anion translocation through the plasma membrane [5–7]. AE1 has been shown to be the sole anion exchanger expressed in erythrocytes and in some kidney epithelial cells [8,9]. AE2 is mainly expressed in choroid plexus, gastric mucosa, intestine and also renal cells that do not express the AE1 isoform [10–12]. AE3 is found in excitable tissues such as brain [6], heart [13] and retina [14].

Moreover, AE1 has been shown to be involved in fish red blood cell volume regulation. The trout anion exchanger (tAE1) has been proposed to mediate the volume-sensitive loss of taurine and other small organic compounds [15,16]. Cloning of tAE1 and expression of this protein in *Xenopus* oocytes led to the conclusion that it forms an anion channel permeable to taurine [17] as well as to some organic solutes (choline, urea and sorbitol [18]) and inorganic cations K^+ and Na^+ [19]. All these permeabilities are activated in swollen erythrocytes by a decrease in intracellular ionic

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strength [20,21]. It appears that tAE1 might bear an anion channel activity which is physiologically involved in cell volume regulation. This anion conductance is spontaneously revealed when the protein is expressed in an heterogeneous expression system. In contrast to fish red cells, mammalian red blood cells do not regulate their volume and lack swelling-sensitive osmolyte channels. Band 3 from mouse erythrocytes (mAE1) and tAE1 are highly similar proteins (71% similarity in transmembrane domains); however, mAE1 expression in *Xenopus* oocyte does not result in increased anion conductance, nor in taurine, polyol or cation transport [17–19].

To define the structural domains involved in the induction of tAE1 anion conductance, we have, in a previous work [17], constructed exchangers composed of a combination of half-transmembrane domains (helices 1 to 5, and helices 6 to 13) of mouse and trout AE1 on each side of extracellular loop no. 5 (Fig. 1). Indeed, a marked difference between tAE1 and mAE1 is the presence of an enlarged extracellular loop (Z loop) between transmembrane segments 5 and 6 in tAE1. Therefore, this loop was chosen to divide the transmembrane domain in two roughly equivalent sections. The construct (trout (1,5)–Z loop–mouse (6,13)) was designated TZM; T and M referring to the species helices originate from, i.e. trout and mouse, respectively (Fig. 2A). Chloride influxes and electrophysiological studies have shown that TZM construct formed a functional anion exchanger when expressed in *Xenopus* oocyte. However, this exchanger did not exhibit ionic current. The reverse construct MZT was not compatible with expression at the oocyte plasma membrane [17]. Whereas it was clear that the C-terminal part of tAE1 transmembrane domain was involved in conductance induction, it was not possible to tell whether it was sufficient since MZT was not expressed.

In this paper, we have expressed several hybrid constructs composed of mouse/trout and skate/trout red cell anion exchangers to go further into our structure–function study. Our results allow us to identify trout transmembrane domains that are involved in tAE1 conductance.

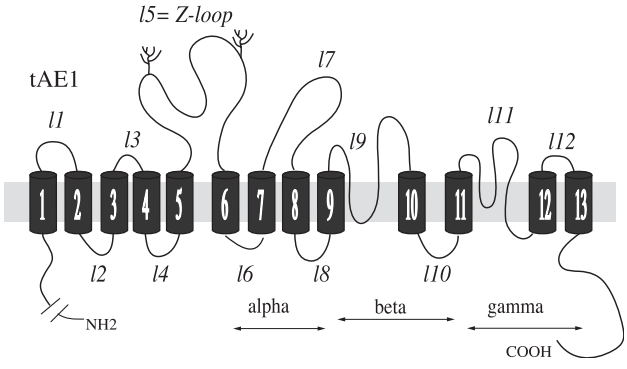

Fig. 1. Schematic diagram of a predicted secondary structure of tAE1 showing zone alpha, beta and gamma analysed in this study. The topology is one described by Zhu et al. [4], with 13 α helices crossing the lipid bilayer, the N terminus and C terminus part of the protein being in the cytoplasm.

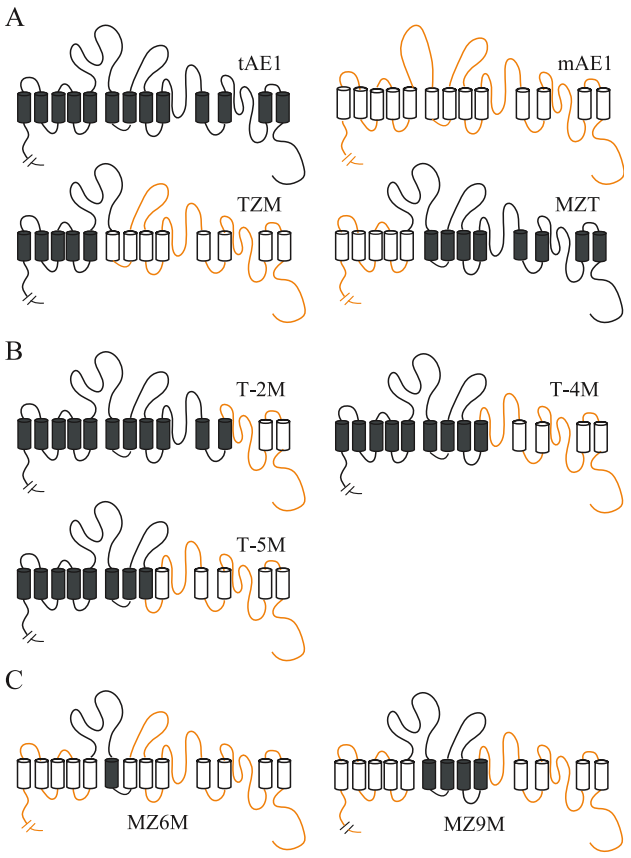

Fig. 2. Schematic representation of constructs studied in this paper. AE1 spanning domains are represented as cylinders. Trout elements are dark, mouse elements are clear. (A) Constructs previously described in Fiévet et al. [17]. (B) Substitution of trout elements for mouse domains. (C) Insertion of trout domains in mouse anion exchanger.

2. Materials and methods

2.1. Oocytes

Xenopus laevis were cooled on ice with MS222 until completely anaesthetized and maintained covered with ice during the surgery according to the procedure recommended by our ethics committee. The surgery consisted of removing about five ovarian lobes containing oocytes. After surgery, the animals were placed in cold water between 0 and 4 °C to recover from anaesthesia, monitored for 3 h and then placed back in their aquaria.

2.2. Chimera construction

The construction protocol of hybrid exchangers has been previously described in Ref. [17]. Anion exchanger SZT was made with half skAE1 and half tAE1. The carboxy-terminal part of tAE1 cloned in pSP64 was amplified in a

PCR reaction with primers SZT-forward and 2919pSP64 (5′-TAGGGCCCATGATTACGAATTCGGTTT-3′) complementary to the cloning vector pSP64. Fifteen bases in the 5′ part of primer SZT-forward were not complementary to trout but complementary to skate sequence in the place where fusion between skate and trout was to be done. Thus, primer SZT-forward (5′-ATC TAC GAA ACC TTC AGC AAG CTC GGC AAG ATC-3′) can be decomposed as follows:

Skate					Trout					
	5′-ATC	TAC	GAA	ACC	TTC	AGC	AAG	CTC	GGC	AAG-3′
	Ile	Tyr	Glu	Thr	Phe	Ser	Lys	Leu	Gly	Lys
a.a. no.	522				526	521				525

The same kind of PCR was used to amplify the N-terminal of skAE1 cloned in pGEMT-easy vector using primers *Hin*dIII-sAE1-14-forward (5′-TATAAAGCTTTAG-GAAGTAAGCACATG-3′) and SZT-reverse. 5′ end of the forward primer (15 bases) was not complementary to skAE1 sequence and contained a *Hin*dIII restriction site.

These two primary PCR products, S and ZT, hybridise in the fusion point region where they overlap. They were then mixed and a secondary PCR was run with primers *Hin*dIII-sAE1-14-forward and 2919pSP64 reverse to amplify the SZT gene fusion. SZT was cloned in pGEMT easy vector (Promega) and fusion was checked by digestion with different restriction enzymes. Finally, SZT was excised from pGEMT-easy using *Hin*dIII and *Eco*R1 and ligated in pSP64 Poly(A) vector.

All chimeras used in this work were constructed following the same process. To avoid a long list of primers, amino acid positions corresponding to junction points will only be given. For instance, the construct SZT described above will be referenced as $S_{526}/_{521}ZT$. Primers can be directly obtained from the DNA sequence 15 mers on both sides of the junction point symbolised by underscored amino acid numbers.

For trout/mouse chimeras, the constructs were: T-2M = $T_{809}/_{825}M$, T-4M = $T_{727}/_{739}M$, T-5M = $T_{692}/_{703}M$. For all constructs, forward primer was TAE519UP (5′-ACCT-TCAGCAAGCTCGGC-3′) using tAE1 as a template, and reverse primer was MB3Bstdw (5′-ATTATTTTCGAA-TTTTCCAGCCTGGGGCC-3′) using mAE1 as template. The secondary PCR product was submitted to *Xho*1 and *Bst*1 digestion and then ligated in *Xho*1/*Bst*1 tAE1-pSP64 poly(A) opened vector.

Construct MZ6M ($MZT_{620}/_{631}M$) was obtained from MZT [17] and mAE1, and construct MZ9M ($MZT_{727}/_{738}M$) as well. For these two constructs, forward primer was M524UP (5′-GTGGCCTTTGAAGGCAGCTTCCTCG-3′) using MZT template, and reverse primer was pSPT19T7 (5′-GCTTATCGAAATTAATACGACTCACTAT-3′) using mAE1 template. The secondary PCR product was *Stu*1- and *Bst*X1-digested and then ligated in MZTpSPT19 (*Stu*1/*Bst*X1) opened vector.

Construct SZT(6)ST(γ) was obtained by a two-step process. First, starting from SZT and skAE1, we got $SZT_{620}/_{604}S$ (SZT(6)S). Then this construct and SZT were used in a second set of PCR reaction to get $SZT(6)S_{791}/_{808}T$ (i.e. SZT(6)ST(γ)).

Construct SZT $_{700}/_{680}$ST(γ) (SZT(α)ST(γ)) was obtained combining SZT(6)ST(γ) and SZT.

Accession numbers for trout, skate and mouse anion exchangers are Z50848, AJ537571 and P04919, respectively. Primer sequences and amplification conditions are available upon request.

All PCR reactions were done with Expand High Fidelity system (Roche) in a Biometra "UNO Thermoblock" thermocycler. All the constructions were checked by sequencing and reading frame integrity was checked by in vitro translation protein assay before injection of cRNA in oocytes (Promega, Transcend non-radioactive translation detection systems).

2.3. Production of cRNA

cRNAs were obtained using a commercial SP6 transcription kit (Ambion). cRNA concentrations were estimated on a formamide/formaldehyde agarose gel in MOPS buffer.

2.4. Oocyte injection

Collected oocytes were washed in Modified Barth's Saline (MBS composition in mM: NaCl: 85; KCl: 1; $NaHCO_3$: 2.4; $MgSO_4$: 0.82; $Ca(NO_3)_2$: 0.33; $CaCl_2$: 0.41; HEPES: 10; NaOH: 4.5; pH 7.4; supplemented with penicillin: 10 U/ml and streptomycin: 10 μg/ml). After washing with MBS, defolliculation was obtained by 16-h incubation at 18 °C in MBS containing between 0.8 and 1.3 mg/ml collagenase (SERVA) corresponding to 1U/ml followed by 30-min incubation in Ca^{2+}-free MBS. Stage V–VI oocytes were then injected with 50 nl of 80 ng/μl cRNA and maintained at 18 °C in MBS. Comparison of water-injected or non-injected oocytes showed no difference regarding Cl^- permeability. Therefore, in experiments presented below, control oocytes refer to non-injected oocytes.

2.5. Western blot of oocyte membrane proteins

Oocyte membranes were prepared by homogenization of 20 oocytes (control or injected) in cooled Tris–HCl buffer, 20 mM pH 7.4, with 0.5 mM of DTT and 0.5 mM of a protease inhibitor Pefabloc (Roche). The mixture was centrifuged at 4 °C; 2000 rpm, then 4000 rpm and finally 6000 rpm in an Eppendorf tube; after each centrifugation, the pellet was discarded. Collected supernatant after the third centrifugation was ultracentrifugated at 65 000 rpm, 30 min, 4 °C and membrane pellet solubilized in the homogenization buffer. Protein concentration was measured with Biorad kit and 50 μg of proteins was then loaded/lane of

SDS-PAGE electrophoresis gel. Western blot transfer was done with a semi-dry transfer system from Biometra on nylon membrane (Hybond C extra, Amersham). The presence of tAE1 was detected by antibody Ab146 directed against a synthetic peptide corresponding to the first 15 amino acids of the N-terminal part of tAE1 (Neosystem, Strasbourg). Immunoglobulins Ab146 were purified from rabbit serum by precipitation with caprilic acid as described by Reik et al. [22]. The secondary antibody was a goat IgG-peroxydase (Sigma, St. Louis, MO) that was detected by chemiluminescence (Super Signal West Pico, Pierce).

2.6. Influx measurements

Chloride influx measurements were done as previously described [19]. Briefly, eight oocytes were incubated at 18 °C in 80-μl MBS containing ^{36}Cl (Amersham) with a specific activity of 360 dpm/nmol chloride. After 15 min of incubation, (linear phase of the uptake kinetic), the oocytes were washed twice in ice-cold MBS and transferred individually into counting vials. The washing procedure took less than 30 s. The volume of extracellular fluid dropped with each oocyte being variable, it was quickly removed and 20 μl of 20% SDS was added before vortexing. Radioactive chloride uptake in each oocyte was determined after scintillation counting with external standard procedure to correct for quenching. The incubation medium was counted in duplicate on 5-μl aliquots, using the same protocol to determine the specific activity in each experiment. Chloride uptake was calculated as the mean of the eight values and expressed as $pmol \cdot min^{-1} \cdot oocyte^{-1}$.

2.7. Electrophysiology

Electrophysiological parameters were measured at room temperature as previously described [17] using the two-electrode voltage clamp technique with a TEV 200 amplifier (Dagan, Minneapolis, MN) monitored by computer through Digidata 1200 A/D converter/PC clamp software (Axon Instruments Inc., Foster City, CA). Current and potential electrodes filled with 3 M KCl had a resistance of 1.8 to 2.3 MOhm. Following resting potential (E_m) determination, oocytes were clamped at holding potential of −30 mV and 800-ms clamping potentials from −100 to +80 mV with 20-mV increments were applied and the current values recorded. I_m was plotted versus V_m and the mean slope of the I/V curve between −100 and −20 mV was taken as an index of the membrane conductance and expressed as micro-siemens (μS).

2.8. Chemicals and reagents

Agarose was purchased from GIBCO BRL (Life Technologies, Gaithersburg, MD). Minipreps of DNA were done with a commercial kit from Qiagen. Unless otherwise stated, all chemicals were purchased from Sigma.

3. Results

To go further into mapping of tAE1 domains involved in the anion conductance, we sequentially exchanged fragments of tAE1 between the carboxy-terminal end and the Z-loop for their mAE1 counterparts. Chimeras were called T-xM, T representing the core of the chimera (tAE1) and x the number of spanning domains exchanged for mAE1 elements. The various constructs are shown in Fig. 2B. We exchanged domains from the carboxy-terminal end to helix 11: T-2M; from the carboxy-terminal end to helix 9: T-4M; and finally from the carboxy-terminal end to helix 8: T-5M. To simplify construct terminology, the region corresponding to helix 6 up to loop 8 (included) has been called "zone α", helices 9 up to 11 "zone β" and loop 11 up to COOH terminal end "zone γ" (Fig. 1). These different chimeras were injected into *Xenopus* oocytes to investigate their functional characteristics. Fig. 3 shows an immunoblot of the different AE proteins. Many Western blots have shown that, even though there was a small discrepancy between experiments, surface expressions of TZM, T-2M, T-4M and T-5M are equivalent. The higher signal in TZM lane was not observed consistently. We can thus conclude that all these constructions were similarly expressed in oocyte. Results regarding chloride fluxes and anion conductance are reported on Table 1. All the chimeras were able to induce a chloride permeability similar to that of tAE1: 306 ± 39 $pmol \cdot oocyte^{-1} \cdot min^{-1}$ (i.e. more than 10-fold the non-injected oocyte chloride influx). It is noteworthy that Cl^- influxes of all the chimeric exchangers are 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS)-sensitive. However, anion conductances of these proteins were greatly different. Replacement of zone γ (T-2M) decreased by two thirds the conductance value: 7.56 ± 1.83 μS for T-2M compared to 28.6 ± 3.1 μS for tAE1. Enlarging the replaced domain to γ+β (construct T-4M) resulted in an even lower conductance: 3.86 ± 1.23 μS. In contrast, replacement of the next helix (T-5M) did not reduce the

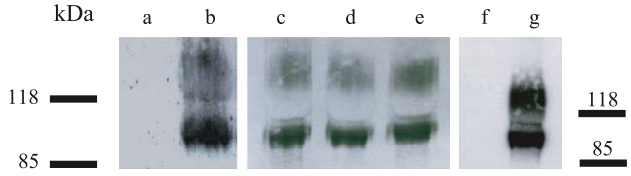

Fig. 3. Western blot analysis of tAE1, T-5M, T-4M, T-2M and TZM expression in oocyte plasma membrane. Membrane proteins of oocytes expressing tAE1 or the different constructions were prepared as described in Section 2. Fifty micrograms of proteins was loaded per lane and immunodetection was made with an antibody directed against the first 16 N-terminal a.a. of tAE1 (Ab146). Lanes a and f correspond to control oocytes (non-injected), lane b to tAE1, lane c to T-5M, lane d to T-4M, lane e to T-2M, and lane g to TZM expressing oocytes. This Western blot is representative of three different ones. The two bands correspond to the glycosylated form of tAE1 (≈ 120 kDa) and the unprocessed form (core-glycosylated); theoretical molecular weight: 100 kDa, as controlled by deglycosylation experiments (not shown).

Table 1
Transport and electrophysiological parameters of oocytes expressing trout/mouse AE hybrid constructs

	Cl^- influx ($pmol \cdot oocyte^{-1} \cdot min^{-1}$)	Conductance (μS)	Percentage of Cl^- influx inhibition by DIDS (0.5 mM)
Control	28 ± 6, $n=104$	0.58 ± 0.02, $n=71$	–
tAE1	306 ± 39, $n=8$	28.6 ± 3.1, $n=37$	insensitive
T-2M	266 ± 31, $n=8$	7.56 ± 1.83, $n=13$	91.5 ± 8.4, $n=8$
T-4M	309 ± 31, $n=8$	3.86 ± 1.23, $n=9$	82.6 ± 6.7, $n=8$
T-5M	373 ± 42, $n=8$	4.53 ± 1.07, $n=17$	81.5 ± 14.2, $n=8$
TZM	261 ± 51, $n=5$	0.40 ± 0.02, $n=8$	88.6 ± 2.3, $n=8$
MZ6M	324 ± 40, $n=8$	0.53 ± 0.03, $n=8$	73.1 ± 18.2, $n=8$
MZ9M	105 ± 9.9, $n=8$	0.48 ± 0.03, $n=8$	90.2 ± 3.0, $n=8$
SZT	282 ± 10, $n=8$	6.08 ± 0.99, $n=8$	83.7 ± 5.3, $n=8$
SZT(α)ST(γ)	275 ± 44, $n=3$	1.46 ± 0.25, $n=3$	63.2 ± 17, $n=8$
SZT(6)ST(γ)	342 ± 43, $n=3$	0.72 ± 0.08, $n=3$	67.4 ± 13.8, $n=8$

Cl^- influx was measured 3 days after injection. Results are mean of n experiments, each of them done with eight oocytes individually counted and then averaged. Conductance values are mean of n different expressing oocytes obtained from independent oocyte batches. Each value has been measured from a linear current–voltage relationship curve portion (− 100 mV and − 20 mM).

conductance: 4.53 ± 1.07 μS. Finally, as already mentioned above, the anion conductance activity was totally null in the chimera TZM. These results clearly demonstrated that zone α (helices 6, 7 and 8 plus loop 8) contained essential elements for the conductive pathway since T-5M was still conductive whereas TZM was not.

The next question to address was whether tAE1-zone α could confer a conductive activity to mAE1. In order to answer this question, two constructs were designed. In the first one (MZ6M), loop 5, helix 6 and loop 6 of mAE1 were exchanged for their trout counterparts. In the second one, the mAE1-exchanged region was extended to zone α: MZ9M (Fig. 2C). These two chimeras were functional in *Xenopus* oocyte. Three days after injection, Cl^- influx induced by MZ6M and MZ9M was 324 and 105 $pmol \cdot min^{-1} \cdot oocyte^{-1}$, respectively. However, these two proteins were not conductive. The anion conductance developed by MZ6M was 0.53 ± 0.03 μS and 0.48 ± 0.03 μS for MZ9M. Thus, we can conclude from these results that zone α was not able on its own to induce any conductive activity. Since zone α was not sufficient, tAE1 zone (α + β) was introduced in mAE1. Unfortunately, the obtained chimera, named MZ11M, was not processed to oocyte plasma membrane, as verified by Western blot (not shown). For the same reason, it was not possible to determine whether zone γ could confer a conductive activity to mAE1. Indeed, the mouse analogue construct of T-2M (M-2T) could not be expressed in oocyte (no Cl^- influx and no immunodetection by Western blot analysis).

We have recently cloned the red cell anion exchanger AE1 of the little skate *Raja erinacea* (skAE1) [23]. Fig. 4A shows the voltage–current relationship of skAE1-expressing oocytes. skAE1 and non-injected oocytes had a similar and very small background current with a slope conductance of 0.58 ± 0.02 μS. skAE1 functioned as an electroneutral exchanger which was not able to induce any conductance when expressed in *Xenopus* oocyte. Therefore, we decided to construct the chimera SZT in replacement of MZT which

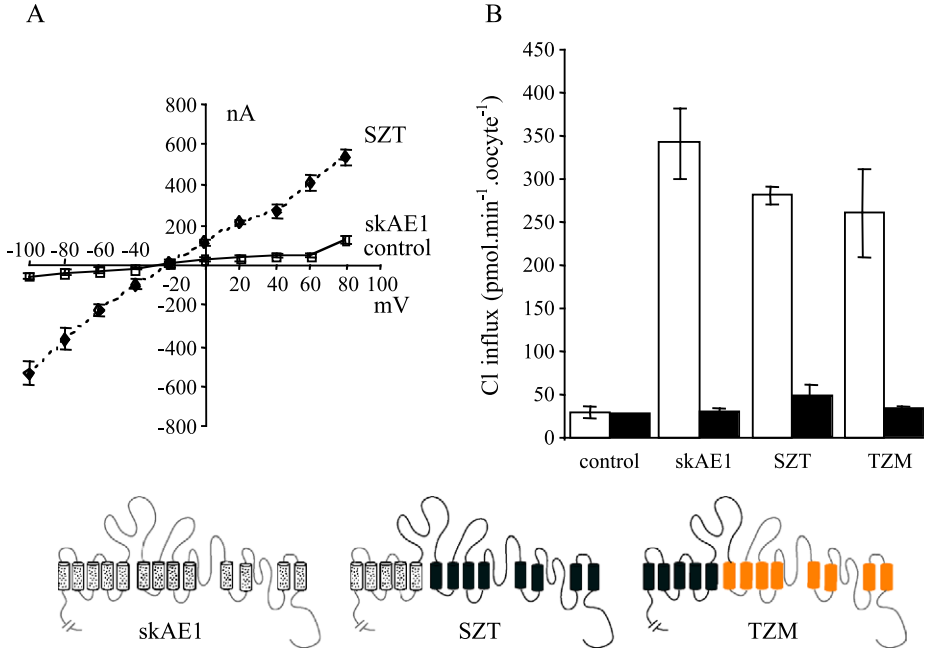

Fig. 4. (A) Current–voltage relationship of skAE1- and SZT-expressing oocytes measured 3 days post injection. Control referred to non-injected oocytes. Results are mean of five different experiments. Currents of each experiment have been individually measured from six oocytes and then averaged. (B) Cl^- influx of oocytes expressing skAE1 compared to different AE1s constructs: TZM and SZT. Control referred to non-injected oocytes. Cl^- influx was measured for 15 min in absence (white bars) or in presence (black bars) of DIDS (5×10^{-4} M). Results are expressed in picomoles of Cl^- per minute per oocyte ($pmol \cdot min^{-1} \cdot ovocyte^{-1}$) and mean of eight different experiments, each of them done with eight oocytes individually counted and then averaged.

was not expressed. It can be seen from Fig. 4B that SZT was fully functional and inhibited by DIDS (5×10^{-4}M). The chloride permeability induced by SZT (282 ± 10 pmol · min^{-1} · ovocyte^{-1}) was equivalent to that of wild-type tAE1 (306 ± 39 pmol · min^{-1} · ovocyte^{-1}) and to that of TZM (300 ± 40 pmol · min^{-1} · ovocyte^{-1}). But the important result is that, in contrast to TZM, SZT exhibited an anion conductance: 6.08 ± 0.99 μS versus 0.58 ± 0.02 μS for control oocytes (Fig. 4A). As TZM was an electroneutral exchanger and SZT exhibited a conductive activity, this definitely demonstrated that zone (α + β + γ) of tAE1 was sufficient to elicit conductive properties in an electroneutral exchanger.

The results mentioned above showed that zones α and γ of tAE1 seemed important for the channel activity. It was then assessed whether introducing tAE1 zones α and γ could elicit a chloride conductance in skate exchanger (skate was chosen instead of mouse to prevent a possible default in protein expression). A new chimera was made: SZT(α)ST(γ) (Fig. 5A). The conductance developed by this exchanger was relatively small, 1.46 ± 0.25 μS, however, it was statistically superior to that of skAE1 (Fig 5B). This conductance could be shut down by reducing the length of zone α to Z-loop plus helix 6: construct SZT(6)ST(γ) was not electrogenic anymore, Gs = 0.60 ± 0.06 versus 1.46 ± 0.25 μS (Fig. 5B). In Fig. 5C, it can be seen that chloride influxes of these two constructs were equivalent, indicating that they were similarly expressed in *Xenopus* oocyte. These chloride influxes were inhibited by DIDS (Table 1). We can then conclude that the entire zone α was necessary to induce minimal anion conductance.

4. Discussion

The amino acid sequences of mouse and trout anion exchanger transmembrane domains are very similar, they share 59% identity (71% similarity) but only tAE1 is able to act as a channel. In a previous publication, we have shown that the C-terminal membrane-spanning domain (from Z-loop up to the carboxy-terminal end) was required to induce conductive activity. Indeed, replacement of this trout protein domain by its mouse counterpart, chimera TZM, totally suppressed conductive activity. The protein TZM was only able to act as an anion exchanger but no more as a conductive transporter [17].

The goal of our structure–function analysis was to insert some elements of tAE1 into mAE1, hoping to transform the latter in an anion conductive protein. However, insertion of trout elements into the mouse exchanger did not allow a correct membrane targeting of the constructs. As previously reported, the chimera MZT is not expressed at the oocyte membrane [17]. This impediment was conserved with MZ11M and partially reversed with MZ9M as shown by the small value of chloride influx in MZ9M expressing oocytes. At least, when the trout portion inserted in mouse

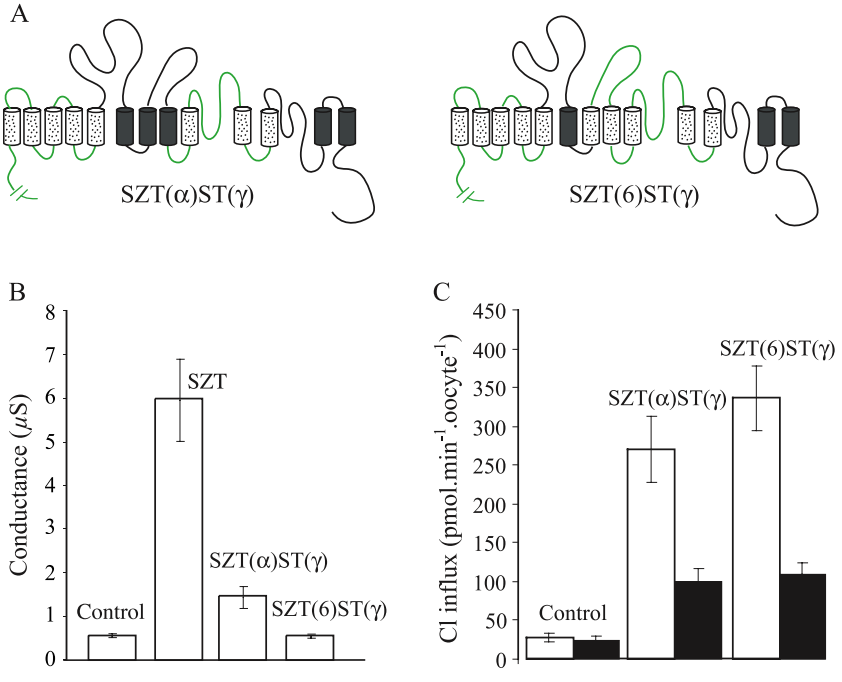

Fig. 5. (A) Schematic representation of two hybrid skate/trout constructs: SZT(α)ST(γ) and SZT(6)ST(γ). AE1 spanning domains are represented as cylinders. Trout elements are dark, skate elements are dotted. (B) Comparison of Cl^- anion conductance of oocytes expressing SZT(α)ST(γ) and SZT(6)ST(γ). Conductances have been measured from a linear current–voltage relationship curve (between −100 mV and −20 mM). Results are mean of three different experiments, measured from eight oocytes and then averaged. (C) Cl^- influx was measured for 15 min in the absence (white bars) or presence (black bars) of DIDS (5×10^{-4} M). Results are expressed in picomoles of Cl^- per minute per oocyte (pmol · min^{-1} · ovocyte^{-1}) and are mean of eight different experiments, each of them done with eight oocytes individually counted and then averaged.

AE was very short (Z-loop + helix 6) the resulting construct MZ6M was correctly expressed.

The difficulty to get correct expression in oocyte with mouse/trout constructs was overcome using the skate AE1 isoform (skAE1) instead of mAE1. skAE1, recently cloned by Guizouarn et al. [23], is more similar to mAE1 (72%) than to tAE1 (66%). In this paper, we showed that this exchanger did not possess the same conductive properties as tAE1; it did not induce any anion conductance when expressed in *Xenopus* oocytes. In contrast to mouse/trout chimera (MZT), the skate/trout construct (SZT) elicited a chloride permeability similar to skAE1 and, interestingly, exhibited an anion conductance (6.08 ± 0.99 μS). This definitely demonstrates that the C-terminal part of tAE1, when fused with the N-terminal part of a non-conductive exchanger, is able to confer a conductive activity to this exchanger.

Based on the topology proposed by Zhu et al. [4], we virtually divided the C-terminal region in three parts: zone α includes the Z-loop and three transmembrane helices (6, 7, 8); zone β contains loop 9 and two helices (10 and 11); and zone γ contains the loop 11 and the last transmembrane domains (Fig. 1). In this paper, we analysed the involvement of these three zones in the anion conductance activity.

From our results, it is obvious that the trout zone γ is greatly involved in the conductive activity since substitution by its mouse counterpart shut down by two thirds the conductance value (28.6 μS tAE1 versus 7.56 μS T-2M). Removal of zone β from T-2M reduced even more the conductance (7.56 μS (T-2M) to 3.86 μS (T-4M)) but the important point is that neither the replacement of zone β nor (β + helix 9) led to the complete disappearance of the anion conductance. In contrast, if zone α was then removed (TZM) the exchanger was turned to an electroneutral transporter, showing that zone α is critical in the development of the anion conductance.

An important question to address was which regions were able to convert a non-conductive anion exchanger to a conductive exchanger. In Fig. 6 are summarised the important points of our analysis. One evidence was that zone α, *alone*, was not able to induce any anion conductance since the exchanger MZ9M (Fig. 6C) was not conductive. Moreover, when zone γ was added to zone α (construct SZT(α)ST(γ)) (Fig. 6D), the exchanger exhibited a small anion conductance. Co-expression of these two zones (α + γ) was able to initiate a conductive function mode in an electroneutral exchanger. The construct SZT(6)ST(γ) (Fig. 6E) gave us an additional element: the integrity of zone α was required to develop a conductance. Indeed, the presence of only (Z-loop + helix 6) instead of the entire zone α impaired the apparition of the anion conductance observed with SZT(α)ST(γ). As one can see, zone α is a crucial element; its presence is necessary but not sufficient to induce conductive activity.

Moreover, results obtained in this study showed that to form a conductive pathway, an interaction between zone α and the first half of tAE1 is required. The first evidence was

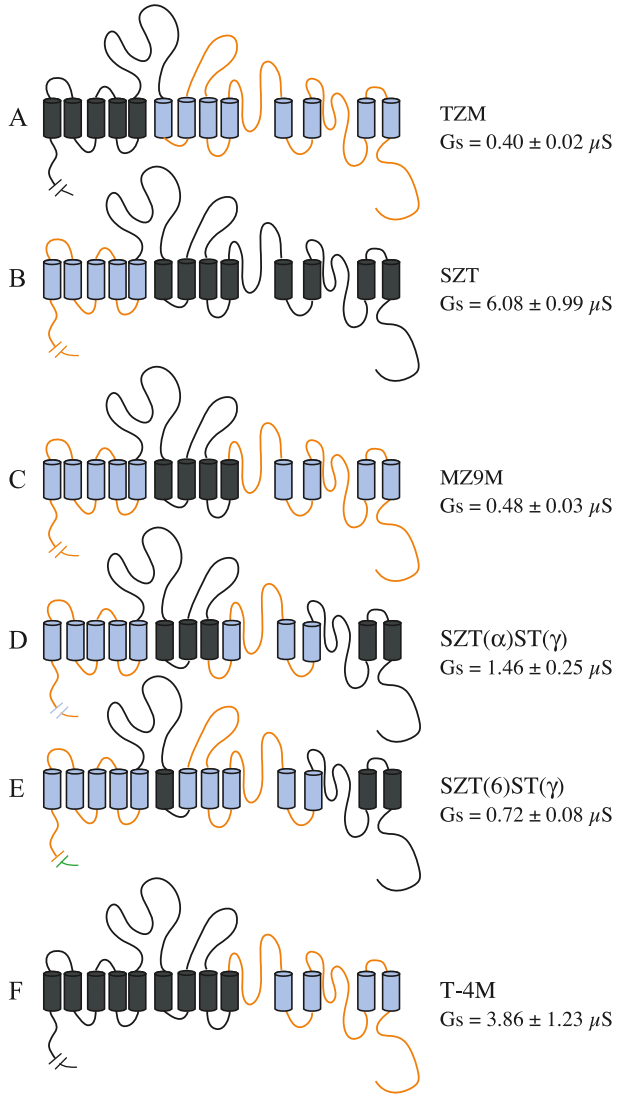

Fig. 6. Plate summarizing the results obtained in this study. Schematic illustration of the constructs are shown on the left part of the plate and the associated conductance values on the right part. Trout elements are drawn in black and mouse or skate elements are drawn in grey.

provided by the comparison of oocyte conductance values induced by two chimeras sharing the same second half of tAE1: MZ9M and T-4M (Fig. 6E and F). MZ9M did not induce any conductance, whereas T-4M (and T-5M) did. Second, oocyte conductance was 28.6 μS when tAE1 was expressed, whereas conductance of SZT expressing oocytes was much lower, only 6.08 μS. These data strongly suggest that all the elements involved in the conductive activity are not restricted to tAE1 C-terminal domain (α + β + γ). Even though this domain is sufficient, the tAE1 N-terminal part is needed to achieve the correct conductive activity.

Although it was impossible to conclude directly on the property of zone γ alone because the exchanger M-2T was not expressed in *Xenopus* oocyte, we can deduce from construct SZT(6)ST(γ), which was not conductive, that zone γ is not able to induce an anion conductance by itself.

In conclusion, when present separately, in an electroneutral exchanger, zone γ and zone α are not able to induce any conductive activity but when present together, they provoke a sudden change in the functioning from an electroneutral exchanger into a conductive transporter. This small conductance needs the presence of zone β and the N-terminal part of tAE1 to get a maximal value. Results presented in this study show that elements which induce conductive activity to tAE1 are not restricted to a small portion of the exchanger, but spread over the whole transmembrane domain. The C-terminal half spanning part contains elements sufficient to install the conductance (zone α and γ) and the rest of the exchanger contributes to the formation of the conductive pathway.

Data reported in this structure–function analysis show that helices 6, 7 and 8 and the putative helices 12 and 13 with connecting loops are linked to tAE1 conductive activity. Alignment of mouse, skate and trout AE1 amino acid sequences shows that most of charged amino acid differences between the trout and the other two sequences are mainly localised in these areas. These amino acids could represent good candidates for point mutation studies. Functional analysis of these mutants will give new elements to find out how, among the several AE isoforms, tAE1 can form a conductive pathway.

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