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Evidence for the Presence of Three Different Anion Exchangers in a Red Cell. Functional Expression Studies in *Xenopus* oocytes

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Abstract. Anion exchangers (AE) are transmembrane proteins catalyzing electroneutral exchange of Cl^- for HCO_3^-. To date, three different genes coding for this protein, AE1, AE2 and AE3, have been identified in many species. AE1 is considered to be the unique anion exchanger expressed in erythrocytes. In this paper we propose the presence of three different AEs in skate erythrocytes, a skAE1, a skAE2 and a skAE3, cloned by RT-PCR (reverse-transcriptase polymerase chain reaction). These three skAE have a similar predicted secondary structure. All three skAE are divided in two main domains: a hydrophilic cytoplasmic N-terminal domain and a C-terminal domain crossing the lipid bilayer at least 12 times. The greatest similarity is found in the membrane-spanning domain of the three skAE. The size as well as the amino-acid sequence of the cytoplasmic domain differ significantly among three anion exchangers. Functional expression studies in *Xenopus* oocytes led to the conclusion that skAE-1 and -2 share some functional features (Cl-dependence and DIDS sensitivity). The skAE3 could not be expressed in *Xenopus* oocytes. These data are in agreement with expression data obtained with AEs of different species utilizing the oocyte system. It is highly probable that these three new AE sequences come from three different genes, thus suggesting for the first time the presence of the three AE genes in Chondrichthyes.

Key words: Anion exchanger — Erythrocyte — Skate — Band 3 — *Xenopus* oocyte

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Introduction

Anion exchangers (AEs) are transmembrane proteins catalyzing the exchange of Cl^- for bicarbonate. 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* (Casey & Reithmeier, 1998; Alper et al., 2002)). All the AE polypeptides can be divided into two main domains of about the same size: a N-terminal cytoplasmic domain and a membrane-spanning domain with a short C-terminal end in the cytoplasm. The isoforms differ mainly in their cytoplasmic domain, the membrane-spanning domain showing great similarities and being responsible for the anion translocation through the plasma membrane (Grinstein, Ship & Rothstein, 1978; Kopito et al., 1989; Lepke, Becker & Passow, 1992). AE1 has been shown to be the unique anion exchanger expressed in erythrocytes and in some kidney epithelial cells (Alper et al., 1989; Alper et al., 1997). AE2 is mainly expressed in choroid plexus, gastric mucosa, intestine and also renal cells that do not express AE1 isoform (Kudrycki, Newman & Shull, 1990; Lindsey et al., 1990; Chow et al., 1992). AE3 is found in excitable tissues such as brain (Kopito et al., 1989), heart (Linn et al., 1995) and retina (Kobayashi et al., 1994).

Up to now, the different isoforms were not found coexpressed in the same cells except in cardiac ventricular myocytes where AE1, AE2 and AE3 are all present, at least at the mRNA level for AE2 (Linn et al., 1995; Puceat et al., 1995; Richards et al., 1999) and also in vascular smooth muscle and intestinal cells where the presence of AE2 and AE3 was reported (Brosius et al., 1997). Associated with their ubiquitous occurrence anion exchangers are involved in many different

processes such as cellular and organismal aging, neurological diseases and structural integrity of the cell. In erythrocytes, the Cl^-/bicarbonate exchanger AE1 is the major membrane protein. It is involved in CO_2 transport by allowing rapid equilibration of HCO_3^- between intra- and extracellular media and it is responsible for the equilibration of Cl^- and H^+ through the plasma membrane of these cells. It also participates in cell shape by anchoring to the cytoskeleton (Jay, 1996) and in glycolysis by interacting with glycolytic enzymes (Harrison et al., 1991; Low, Rathinavelu & Harrison, 1993). In fish red cells, the anion exchanger has also been proposed to participate in cell volume regulation mediating the volume-sensitive loss of taurine and other small organic compounds (Garcia-Romeu et al., 1996; Perlman, Musch & Goldstein, 1996). Cloning of tAE1 (trout anion exchanger 1) and expression of this protein in *Xenopus* oocytes led to the conclusion that it forms an anion channel permeable to taurine (Fievet et al., 1995) as well as to some organic solutes (choline, urea and sorbitol; Fievet et al., 1998) and inorganic cations K^+ and Na^+ (Guizouarn et al., 2001). All these permeabilities are activated in swollen erythrocytes by a decrease in intracellular ionic strength (Motais, Guizouarn & Garcia-Romeu, 1991; Guizouarn & Motais, 1999). In skate red cells, biochemical as well as pharmacological data suggest that the anion exchanger participates in the swelling-induced loss of taurine and organic osmolytes (Goldstein & Brill, 1991; Goldstein & Davis, 1994; Musch, Leffingwell & Goldstein, 1994). In both cell types the anion exchanger is involved in the regulatory volume decrease response (RVD). However, comparison of permeabilities activated by swelling of either skate erythrocytes or trout erythrocytes points out some differences. For instance, skate erythrocyte swelling does not induce any increase in K^+ or Na^+ permeability, as is observed in trout erythrocyte (Garcia-Romeu, Cossins & Motais, 1991). The purpose of the present study was to clone the anion exchanger of skate red cells to further investigate its specific transport features. Surprisingly, skate erythrocytes express three different AE RNAs coding for an AE1, an AE2 and an AE3. It is the first time that three isoforms of anion exchangers are found in a red cell and it raises the question of the functions of three different isoforms of AEs. This paper presents cloning of the three skAE isoforms and functional expression studies of these proteins in *Xenopus* oocytes. Comparison with data available on other AEs is then made.

Materials and Methods

Tissue Removal

Little skates (*Raja erinacea*) caught in Frenchman's Bay (Maine) were bled as follows: 5 ml of blood was drawn from caudal vessel using a heparinized syringe, on skates maintained in sea water. The whole brain was removed from fish anesthetized with MS222 in seawater.

RNA Purification

Blood (hematocrit averaging 21%) was centrifuged and washed three times with 940 EIM (composition in mM: NaCl 300, KCl 5.2, $MgSO_4$ 2.7, $CaCl_2$ 5, Urea 370, TrisHCl 15, pH 7.5) to remove the buffy coat and collect erythrocytes. The absence of white cells was assessed by microscopic observation of the blood after two washes. RNA was isolated as previously described. Briefly, 1 ml of packed red cells (or 1 mg of skate brain tissue) was lysed in 10 ml of D solution (guanidinium thiocyanate 4 M, Na-citrate 25 mM pH 7, sarcosyl 0.5% wt/vol, 2-mercaptoethanol 0.1 M; Chomczynski & Sacchi, 1987) and homogenized for one minute before extraction with phenol:chloroform:isoamyl alcohol (100:49:1) in Na-acetate buffer of pH 4. The aqueous phase containing total RNA was then collected and precipitated by addition of an equal volume of isopropanol and incubated at −20°C for two hours. After centrifugation of this suspension, the RNA pellet was disolved in DEPC-treated water and stored at −20°C before purification of mRNA.

mRNA Purification and cDNA Synthesis

PolyA RNAs were purified by affinity chromatography with oligodT using a commercial kit (Oligotex mRNA kit, Qiagen, Chatsworth, CA). PolyA RNA was retrotranscribed into cDNA with AMV reverse transcriptase and an oligodT primer, according to the instructions from Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Double-stranded cDNA was then used for degenerate PCR.

Degenerate PCR

Degenerate PCRs were run on 25 ng of cDNA from skate red cells with 4 pairs of degenerate primers located within the spanning domains of anion exchangers. Forward primers: AE402, AE455; reverse primers AE712 and AE799. PCRs were done in 50 µl containing 3 µM primers, 200 µM dNTP, 1× buffer Expand High Fidelity, 1.75 units Expand High Fidelity enzyme (Roche Molecular Biochemicals, Indianapolis, IN) and samples were then covered with a drop of mineral oil. PCRs were done with a Perkin Elmer DNA thermal cycler 480. Samples were denatured at 94°C for 2 minutes prior to addition of Expand High Fidelity Polymerase and then cycled for 5 times using a cycle of 94°C—30 s, 52°C—45 s, 72°C—1 min, followed by 15 cycles of 94°C—30 s, 50°C—45 s, 72°C—1 min and 15 cycles of 94°C—30 s, 49°C—45 s, 72°C—1 min. Each PCR, analyzed on agarose gel, gave only one major product that was ligated, after gel purification, in pGEMT easy vector (Promega, Madison, WI) prior to transformation of Top10F competent bacteria (Invitrogen, San Diego, CA). Recombinants were screened using white/blue selection and 13 positive clones were sequenced.

RACE (Rapid Amplification of cDNA Ends) PCR

Adaptors were ligated to a cDNA library of skate red cells or of skate brain according to instructions from Marathon cDNA amplification kit (Clontech). All the RACE PCRs were done using a Biometra Uno or a Perkin Elmer DNA thermal cycler 480 with Expand High Fidelity enzyme (Roche). A typical 50 µl RACE PCR reaction contained: approximately 1 ng of adaptor-ligated cDNA, 200 µM dNTP, 200 nM adaptor-primer (AP1 or AP2), 400 nM skAE

specific primer, Expand High Fidelity buffer 1×, 1.75 units Expand High Fidelity enzyme (Roche). RACE PCR products were cloned in pGEMT-easy vector (Promega) for further sequencing.

skAE1

5'RACE PCR was run on skate red cell cDNA with AP1 (primer for adaptor part of cDNA) and AE651 primer specific of skAE1 isoform, then reamplified in a secondary nested 5'RACE PCR using AP2 and AE73 primers. This yielded 5' products of about 500 bp. 3'RACE PCR was run with AE422 specific forward primer of skAE1 and AP1 as reverse. This RACE PCR gave a major product of 5 kilobases, which was purified on an agarose gel using the Qiaquick gel extraction kit (Qiagen) and amplified in a nested 3'RACE PCR using AE752 and AP2 primers. It gave a major band of 4 kb. Only the first 1000 bp (among which 342 bp were coding) of two clones were sequenced.

skAE2

To identify the 5' end sequence of skAE2, three different 5'RACE PCRs were done on adaptor-ligated cDNA of skate red cells. Each of the PCRs gave a part of the N-terminal domain of skAE2. About 700 bp were obtained with AP1 and AE344 primers. Then 5'RACE PCRs with AP1 and AE158 primers, reamplified using nested primers AP2 and AE67, gave 1 kbase incomplete N-terminal products. The third 5'RACE PCR was done with AP1 and AE182, then reamplified in a nested PCR using AP2 and AE98. It gave 1.6 kbases containing 5' non-coding (about 300 bp, the length of non-coding parts depending on the clones) and 5' coding parts of skAE2 (1251 bp). 3'RACE PCR was done on skate red cell cDNA with AP1 and AE358. This reaction was then diluted 500 times in a secondary nested 3'RACE PCR with AP2 and AE711. The obtained products were about 1 kbase and contained the 3'coding part of skAE2 (670 bp) with partial non coding sequence (without polyA-tail) between 188 and 273 bp, depending on the clones.

skAE3

RACE PCR made with cDNA of skate erythrocytes failed to give specific products. Thus it was decided to clone the 5' and 3' ends of skAE3 in a cDNA library of skate brain, hypothezing that this tissue expresses predominantly the AE3 isoform. To get the N-terminal part of skAE3 (2100 coding bp), four different 5'RACE PCRs were needed. The first one was done with AP1 and AE468 specific reverse primer, this reaction diluted 2500 times was then reamplified in a nested PCR with AP2 and AE333 giving products ranging from 0.6 to 1 kb, corresponding to an incomplete 5' end of skAE3. One other set of 5'RACE PCR with AP1 AE333 and then AP2-AE(3)182 yielded 1 kb. Two other 5'RACE PCRs were needed to finally know the complete N-terminal part of skAE3, using first specific primers AE304 and AE244 and then primers AE217 and AE161. 3'RACE PCR was done with skAE677 and AP1 primers and this reaction 500 times diluted was reamplified with AE804 and AP2. It gave a main product of about 600 bp, corresponding to the 3'coding end of skAE3 (544 bp) and about 40 bp of non-coding nucleotides without polyA tail.

Cloning of Full-length cDNA

Full-length skAE2 was generated by PCR on 2 ng of skate red cell cDNA with AE291 as forward primer (starting 10 bp upstream first ATG) and AE4004 as reverse primer (starting 46 bp downstream the first stop codon). The 3714 pb product was cloned in pGEMTeasy vector and one positive recombinant clone was entirely sequenced.

To clone skate AE3 in red cell cDNA, two primers (AE3for and AE3bfor) corresponding to the 5' part of brain skAE3 overlapping the starting codon and two primers corresponding to the 3' part overlapping the first stop codon were used. The PCR was done on 4 µl of skate red cell cDNA and 30 cycles of 94°C—30 s; 67°C—30 s; 72°C—3 min were applied. This first PCR, 50 times diluted, was reamplified with nested primers (AE3bfor and AE3brev) using the same cycling procedure. A full-length cDNA of skate red cell AE3 was obtained and cloned in pGEMT easy vector for sequencing analyses.

Full-length skAE1 was generated by taking advantage of the presence of unique restriction sites within overlapping sequences of skAE1 in three clones of pGEMTeasy vectors containing: the first one, the 5' end of skAE1 (≈340 bp); the second one, the middle part of skAE1 (2156 bp) and the third one, the 3' end of skAE1 (≈500 bp). Ligation of the three parts of skAE1 gave a full-length skAE1 with a large non-coding domain in 5' and 3' (Fast link DNA ligation kit, Epicentre technologies). Then a PCR was done on this pGEMTeasy vector-skAE1 construct with primers AE129 forward (25 nucleotides upstream starting codon) and AE2869 reverse (20 nucleotides downstream first stop codon) to remove most of the non-coding nucleotides. This PCR product of 2740 bp was cloned in pGEMTeasy vector and one of the positive recombinant clones was sequenced completely before further use.

Sequencing

All PCR products were sequenced using the Big Dye Terminator mix (PE Applied Biosystem, Foster City, CA). Sequencing was performed using a Perkin Elmer GeneAmp PCR system 2400. Samples were run on an Applied Biosystems 377 sequencer. DNA sequences were compiled using DNA sequencing software version 2.1.1 from ABI. For each positive RACE PCR product, between 3 and 6 different recombinant plasmids were sequenced two times from both sides. For degenerate PCR products, two different clones, corresponding to each of the three isoforms, were sequenced three times on both sides, using different primers. Full-length cDNAs corresponding to each of the three skAEs were also entirely sequenced in pGEMT-easy vector and in pSP64polyA vector after subcloning, using different primers. Data-base searches were performed using the FASTA or BLAST programs.

Subcloning in pSP64polyA

The three skate AEs were subcloned in pSP64polyA vector (Promega) since it was observed that cRNAs transcribed from pGEMTeasy vector were less efficiently translated in *Xenopus* oocyte than cRNA transcribed from pSP64polyA vector. Inserts of skAE2, skAE1 and skAE3 were removed from pGEMTeasy vector with appropriate restriction endonucleases (New England Biolabs) and ligated in pSP64polyA vector prepared from digestion of our pSP64polyA-tAE1 construct, using the Fastlink DNA ligation kit (Epicentre technologies-TEBU).

cRNA Synthesis

Apa*I*-linearized pSP64polyA-skAE1 plasmid, EcoR*I*-linearized pSP64polyA-skAE2 and pSP64polyA-skAE3 plasmids were transcribed by SP6 RNA polymerase using the Ambion transcription kit. cRNA concentrations were determined on a formamide/formaldehyde agarose gel in MOPS buffer.

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 three hours and then placed back in their aquaria.

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 hours incubation at 18°C in MBS containing between 0.8 and 1.3 mg/ml collagenase (SERVA) corresponding to 1PZU/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. The 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.

Influx Measurements

Chloride influx measurements were done as previously described (Fievet et al., 1995; Guizouarn et al., 2001). 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 different incubation times (*see* figure legends), the oocytes were washed twice in 8 ml ice-cold MBS and transferred individually into counting vials. 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 8 values and expressed as pmol/min.oocyte.

Chemicals and Reagents

Agarose was from GIBCO BRL (Life Technologies, Gaithersburg, MD). Minipreps of DNA were done with a commercial kit from Qiagen. Unless stated otherwise, all the chemicals employed were purchase from Sigma (St. Louis, MO). Plasmid pSP64 polyA-tAE1 was made in the laboratory (Fievet et al., 1995). Primers were obtained from Eurogentec (Belgium) or Invitrogen. Sequences: AE799: CCCAT(G/A)TAIA(G/A)(G/A)AA(G/T/A) ATICC(G/A) AA; AE712: TCIA(G/A)(G/A)TG(G/A)AAICCI(G/C)(A/T)ICC (T/C)TT; AE402: TA(C/T)TT(C/T)GCIGCI(C/T)TI(T/A)(G/C) ICCIGC; AE450: CCI(C/T)TI(C/T)TIGTITT(C/T)GA(G/A)GA (G/A)GCITT; AE78: CA(A/G)GCITA(C/T)GTIGA(A/G)CTIAA (C/T)GA.

Specific primers for skAE1: AE651: TGCCACTTGGCGGA AAACCTTG; AE73: TCCATCGTGCACGGTCCTTCCA; AE422: ACAACACAAGTGTGAATGGTACAA; AE752: TGCC TGCCTCAATTGTTCCCGCCA; AE129: GTTGGAGTGCGT TTAGGAAGTAAG; AE2869: GAATGGGTTTGGCGTCGTT AGGAT.

Specific primers for skAE2: AE344: CCTGTCCGCCTCACG CCCTC; AE158: AGCGGGAGATGAACCGCACGA; AE67: CGGTCAGGTATTCCATTCCGTTCGA; AE182: GTGGGCCA TCAACGAGCCCAGG; AE98: GAGGAGCAGTGCACGGA GAACG; AE358: GCGCTCCTGTCGCTGGTCCTC; AE711: CATCAGCAAGAAGGAGCGGATGTT; AE291: AGGTGA CACGATGGACTTGCC; AE4004: CTCCATTCCTGCCGGTT-CAGT.

Specific primers for skAE3: AE677: TGATTGTGAGCAAGA AAGAACG; AE804: GAGAAGCATCGCTCACGTCAACG; AE468: CATCACCAGGATGGCAATCGGA; AE333: CAGCG CCGTGTTGGGTTGCATAA; AE288: GTTGCCCTCCAGC TCCGGGAA; AE(3)182: ATCTCCAACACGGACTCCTCC; AE304: CTCCTGCCACGTCATCTCGTTGTT; AE244: CTCGT GAGGCTTCCTGTCGAGCTT; AE217: AATTGTCTCGTGCA TCCAGAGAGC; AE161: TCGGGTGGTGTTGGTGTCCGC; AE3for: CTCACCGTGACCATGGCAACCA; AE3bfor: TGAC CATGGCAACCAGCGGCAG; AE3rev: GCTGGTCTCCATCA CTGGGGC; AE3brev: CCATCACTGGGGCATGTGCATCTC.

Results

Cloning of Three Anion Exchanger Isoforms in Skate Red Cells

In previous studies, the anion exchanger of skate red cells (skAE) was immunoprecipitated and micro-sequenced over 30 amino acids in the N-terminal part of the protein (Musch, Hubert & Goldstein, 1999). This skAE peptide sequence was compared to the cytoplasmic domain of the trout anion exchanger 1, tAE1 (Fievet et al., 1995). Both sequences were identical over the compared amino acids. Thus, a degenerate primer (AE78) was designed corresponding to this motif, aa78 to aa86 of tAE1 sequence and used as forward primer in PCR on cDNA of skate red cells. Moreover, the amino-acid sequences of thirteen previously cloned anion exchangers from different species and tissues were aligned and the comparison of these sequences allowed identification of highly conserved transmembrane domains. These conserved amino-acid sequences were used to design four degenerate primers (*see* Materials and Methods: two degenerate sense primers [AE402 and 455] within the first and the third putative span and two degenerate antisense primers [AE712 and 799] in putative spans 9 and 11). Four different combinations of degenerate primers within the membrane-spanning domain were used in PCR reaction with cDNA from skate red cells. Agarose gel analysis of these PCR reactions showed that each pair of primers gave only one major band of DNA that was cloned in pGEMT-easy vector (Fig. 1). Analyses of 13 clones of pGEMT-easy vector recombinant for different PCR products showed that the same pair of degenerate primers within the spanning domain amplified two different anion exchanger sequences in skate erythrocytes, and primers AE78-AE799 amplified only one single anion-exchanger sequence. Three different AE sequences were obtained with about the same frequency in the different degenerate-PCR. The nucleotide sequences of these three different pieces of anion exchangers shared no more than 66% identities and alignment of these sequences did not give evi-

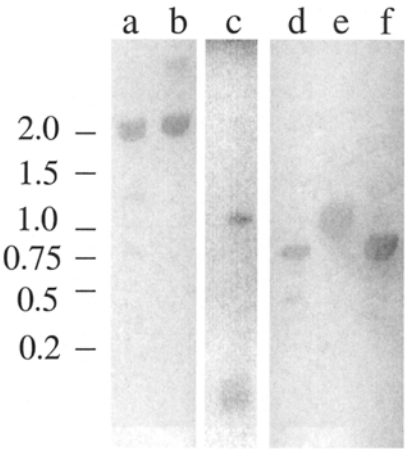

Fig. 1. Agarose gel electrophoresis of degenerate PCR products obtained on skate red cell cDNA. Lane *a* corresponds to primers AE78 and AE712; lane *b* corresponds to primers AE78 and AE799; lane *c*, to primers AE450 and AE799; lane *d*, to primers AE450 and AE712; lane *e*, to primers AE402 and AE799 and lane *f*, to primers AE402 and AE712. Molecular weight markers (in kb) are plotted on the left of the picture.

dence for any alternative splicing possibility. Thus, specific primers for each sequence were used to identify 5′ and 3′ ends of the exchangers (*see* Materials and Methods section).

Except for one AE that was easily amplified over 2156 bp with degenerate primers and whose 5′ and 3′ coding ends where also easily cloned by RACE PCR, cloning of the two other full-length AEs was done on cDNA of skate erythrocytes using pairs of primers specific for each AE. The obtained PCR products were sequenced two times on both sides with different primers and compared to the corresponding 5′RACE, 3′RACE and degenerate PCR sequences. Whenever there was any doubt about any sequence, a region would be sequenced with multiple primers to confirm.

The comparison of the obtained full-length sequences with available AE sequences led to the conclusion that there was an AE1, an AE2 and an AE3 isoform. The nucleotide sequence obtained for skAE1 was 3316 bp long and contained an open reading frame of 2694 bp with 155 non-coding bp in 5′. The non-coding 3′ end was over 4 kb and was sequenced for 467 bp after the first stop codon. The longest sequence identified for skAE2 was 4139 bp in length, 188 non-coding bp in 3′ and 300 non-coding bp in 5′. The cloned full-length skAE2 had an open reading frame of 3651 bp. The cloned skAE3 sequence was 3665 bp long with an open reading frame of 3657 bp. The skAE3 isoform is also expressed in skate brain and was cloned in this tissue before we could clone it in skate erythrocytes. Figure 2 is an alignment of the amino-acid sequences of the three skAEs cloned in skate red cells.

The skAE1 predicted polypeptide is composed of 898 amino acids with a relative mass of 98 kDa. The hydropathy profile is very similar to that of other AE1 isoforms with about half of the protein in the cytoplasm (392 aa N-terminal part) and half of the protein in the membrane (506 aa). skAE2 protein is composed of 1217 amino acids and the hydropathy profile shows an extensive hydrophylic N-terminal domain of 695 aa and a membrane-spanning C-terminal domain of about 523 aa. skAE3 is composed of 1218 amino-acids divided in two main domains, like other members of the AE family: a N-terminal cytoplasmic hydrophylic part of 707 amino acids and a C-terminal membrane-spanning domain. The ORF of these three skAEs were confirmed by in-vitro transcription (*data not shown*).

Table 1 summarizes amino-acid comparisons between these three new AEs sequences and already known human AEs sequences. Comparison of full-length proteins showed that skAE2 is closer to hAE2 than to hAE3 and it was also the case when it was compared to other mammalian or chicken AE2s or AE3s. About skAE3, it is the only known AE3 sequence from a vertebrate other than mammals. Comparison of this sequence with hAE3 or hAE2 indicated more similarities with human (and other mammalian) AE3 (73.4%) than with hAE2 (69.6%). skAE1 was only compared to other AE1 isoforms over full-length protein. This analysis shows that skAE1 shares more similarities with hAE1 (72.4%) than with its fish counterpart tAE1 (trout anion exchanger 1) (67%, data not in the table). Moreover, a phylogenic analysis of these sequences undoubtedly classified skAE3 with other AE3s, skAE2 with other AE2s and skAE1 with other AE1s (*data not shown*). When the comparison of amino-acid sequences is limited to the putative membrane-spanning domains of these proteins, the percentage of similarities and identities increases. As for all AE1, the N-terminal cytoplasmic domain of skAE1 has higher variability than the spanning domain. There is 82.9% similarity between skate and human AE1 in the membrane-spanning domain and 55% in the cytoplasmic domain. Between trout and skate AE1, there is 75% similarity in membrane-spanning domain and 56% similarity in cytoplasmic domain. In the transmembrane domain, the presence of a large extracellular loop found in trout AE1 (called Z-loop) connecting span 5 and 6 in tAE1 is not found in skAE1. However, the band 4.1 binding site (IRRRY) is present in skAE1 as in all AE1, but in skate AE1, the first arginine residue is substituted by a lysine (IKRRY). There is also the cluster of acid residues between E92 and D100. A noticeable tyrosine, Tyr7, is found in skAE1 at the same position as Tyr8 in hAE1. In hAE1, this tyrosine residue is a substrate of phosphorylation by p72syk (Brunati, 2000). There are many putative glycosylation sites in extracellular loops connecting transmembrane helices of skAE1: three are located in the loop connecting spans 5 and

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sAE2              MDLPGSPEIHAAVQGSEQGALPVGPLRIEDEEEED--LNKTWGVERFDDL   48
sAE3      MATSGSKDPPRVLTCSPLAEDEVEGPEATEVVGEEEEEEEEEEEDEDLDRLLPVDHLGDL   60
                     *. **  .  *:*.*   :      . *:*****  *::   *::::.**

sAE2      LHDASSRCVDEVRRTFDEADFEYHRHSSLHTHHPLSTHMP-----------SEGRRKKGT   97
sAE3      LSDLPR---PQPERTYNEKDFEYHRRSSLHIRHPLSARLPPHVCIRRKVRHSERKRRRKR  117
          * * .     : .**::* ******:**** :****:::*           ** :*::

sAE2      QKKSKKTASSPTGAPTIDEGDEDDDHSDGTQEKKEPAEGGMQPDAAANVQFFMPDEELEN  157
sAE3      RKKQRTSLPPSGVTPTIQEVDEEEEHGEGDGSEEE------ARDKEVSASERTRDSPTSH  171
          :**.:.: ...  :***:* **:::*.:*  .::*        *  ....    *.  .:

sAE2      NAASLSPPDRSPKQQDVPSPLSEPGIILPHSHLGRSSERMALRAAAVSAPSLLEENRVLL  217
sAE3      LASQESTESLLIEERTSTPPE---GPMEPQ----RESTQSSLDARDNSPPSCAKR-RSLH  223
           *:. *. .   :::  ..*    * : *:    *.* : :* *   *.**  :. * *

sAE1         MAHRGSYKLQ-MLATGEESPKPHSRFISGTADPSRGKIILQ-----------KDPPG   46
sAE2      KTPLTHRSSYDLKERRRIGSQTNVDEAKFQKIPTDELEAEMLAVADLDYMKSHRFEDVPG  277
sAE3      RTPGAQRPRYDLQERVCIGSMTASGAAIFQKVPTDEAEAEMLAVVDLVYMKSHRFEDHPG  283
          :** ::*. *.*:     *. :    : * . .:*  ..:::             :* **

sAE1      T------LPFQSDIYHKNFG-----------KETFHKAYVEMNELTKDG-EKSYWKDRAR   89
sAE2      VRRHLVRRSNKGTVVHVSKDQSELYPKAKKPDRKPLEVFVELNELTMDKNHEMQWKETAR  337
sAE3      VRRHLVKKPSKGQMSKNTSKMHQTGPRKSKLDRKPHEVFVELNELVVDKNNEMTWQETAR  343
          .      .  :. : : ...           ...  :.:**:***. *  .:  *:: **


sAE1      WIMYEEKLEEETDRWSKPHVPCLSFRYLLEVQRAINNGAVLFDLKETTMSGISVHIVEEM  149
sAE2      WIKFEEDVEEETDRWGKPHVASLSFRSLLELRKTLAHGTVLLDLDQRTLPGIAHQVVEQM  397
sAE3      WIKFEEDVESETDKWGKPHVASLTFRSLLELRKTIAQGAVLLDLEQKTLPGIAHLVVETM  403
          ***:**.:*.***:*.****..*:** ***:::::: :*:**:**.: *:.**:  :** *

sAE1      IAKDQIRAQMRITVLNTLLLKHRHQFDEVRGHLDIETKLKEG------------------  190
sAE2      IISDQIKAEDRANVLRALLLKHCHPNKDKDFSFSRNISTASLGSLMAHHQSQNHIAPSSE  457
sAE3      VISDQIKAEDRANVLRALLVKHSHPNDEKEGSFFPRTGSSSVSSVLGNHHLHHEHYPSND  463
          : .***:*: * .**.:**:** *  .:    :  .    .

sAE1      -------------------------------------HQSRRHSEIMDKVPENTEATLV  211
sAE2      PSVTQPLMGE----STDADVKVMVDSIEKECPLSEMARSRSKHELKLMEKIPCNAEATVV  513
sAE3      QVITLPLMEEERAQSDDSKTLESDSQNTARHTSQQSERHMTKSEIKLLRQIPDNAEATVV  523
                                                :: . ::: ::* *:***:*

sAE1      LVGCVETMASPAMAFVRLEQAVHLESVLGIPIPTRFIAVFLGPSTSNLDYHEIGRSLSTL  271
sAE2      LVGCVEFLEHPTMAYVRLQTAVELESVLEVPVPVRFLFILLGPTNSNMDYHEIGRSISTL  573
sAE3      LIGTLAFLEQPTMAFVRLSEGVLLDSVLEVSVPVRFIFVLLGPSQSNIDYHEIGRSISTL  583
          *:* :  :  *:**:***. .* *:*** :.:*.**: ::***: **:********:***

sAE1      MADKVFRQVAYEAKSRCDLLTGIREVLDYSIVISPTEVHDEDLLRSVIPFQHEMLLKRKN  331
sAE2      MSDKNFHRAAYQADDRNDLLNAINEFLDSSIVVPPSDVPGEELLLSVAQFQRKMLKKRQA  633
sAE3      MSDKEFHEAAYLADDRQDLLNAINEFLDCSIVIPPLELEGRDLLRSVTSFQKEMLRKRRE  643
          *:** *:..** *..* ***..*.*.** ***:.* :: ..:** **  **::** **:

sAE1      KIDKKLGKTAVP-LQIAARKTTPPE--DDDPLKRTGRPFGGLIRDIKRRYPKYLSDIKDA  388
sAE2      KEEQVLSKEVKSQEERALLKRKRLD--EDDPLRRTGKPFGGVIRDAARRYPQYLSDFKDA  691
sAE3      RELKKSLKKEESVLEIKDMCVELREDVEVDSLERTNRLFGGLIRDVRRRYPQYLSDIRDA  703
          :  :   *   .  :          :  : *.*.**.: ***:***  ****:****::**
            ________________________              ____________________
sAE1      LNTQCLAAIIFIYFAALWPAITFGGLLEEKTKGNMGVSELIVSTAVQGIVCCLIGAQPLL  448
sAE2      LNPQCMAAIIFIYFAALSPAITFGGLLGEKTMGLIGVSELIISTAIQMIIFGLLGAQPLL  751
sAE3      FNVQCLAAIIFIYFAALSPAITFGGLLGAKTEGLIGVSELIVSTSIMGIVFSLLGAQPLL  763
          :* **:*********** *********  ** * :******:**::  *:  *:******
```

Fig. 2. Continued on next page.

6 and one is located in the loop connecting spans 7 and 8.

As for other AE2 isoforms, skAE2 has a long extracellular connecting loop between spans 5 and 6. This new AE2 is about the same size as chicken AE2 and a little bit shorter than human AE2 (1219 aa for chicken AE2 and 1240 aa for hAE2). skAE2 shares 78.9% similarity with cAE2 and 77.7% similarity with

```
sAE1    ILGFSGPLLVFEEAYFNFCETLGVDYLAGRVWVGFWMIIIVVVLVAFEGSFMVRFISRFT  508
sAE2    IVGFSGPLLVFEEAFFKFCDSNGMEYLTGRVWIGFWLIAIVLLIVAFEGSFLVRFISRFT  811
sAE3    IIGFTGPLLVFEEAFYNFSKDQHIDYLTGRVWIGFWLMFIVLITVALEGSFLVRYVTPFT  823
        *:**:*********:::*..   ::**:****:***:: **:: **:****:**::: **

sAE1    QEIFSILISLIFIYETFNKLFKIFKAHPLTEHYNTSVNGTIVSGTNLTP----------  557
sAE2    QEIFASLISIIFIYETFYKLVKIFKDHPLRGCSNSTLNGTFLPLIPPSPSNETTEGVRRT  871
sAE3    QEIFAFLISAIFIYETFKNLLIVFAAHPLLKSYPVFPELEGNVTKDLVV----------I  873
        ****: *** ******* :*. :*  ***        :

sAE1    -QPNTALLSLVLMLGTFFIAIFLRKLKTSQFLPGTIRRVIGDFGVPIAILSMVLVDFFIK  616
sAE2    GEPNTALLSLVLMAGTFFIAFFLRKFKNSRFFPGRVRRVIGDFGVPIAILIMVLVDYSIQ  931
sAE3    PMQNTALLSLVLMVGTFFTAYFLRKFRNNRFLAGSVRRTIGDFGIPIAILVMLLVDISVP  933
         :********** **** * ****::..:*:.* :**.*****:***** *:***  :

sAE1    T-FTQKLNVPDGLQVTNYQNRTWFIHPLEG---IPYWMMPASIVPAMLVLILIFMESQIT  672
sAE2    ETFTQKLSVPTGLSVTSPEKRGWFINPLGKNGDFPIWMMFASVIPAILVFILIFMETMIT  991
sAE3    DTYTEKLNVPLHFSVTAPEKRRWFIHPLGKDNGFPIWMIFGAAIPAILLFILIFMETNIT  993
          :*:**.**  :.**  ::* ***:**     :* **: .: :**:*::******: **

sAE1    TLIVSKPDRKMVKGSGFHLDLLLIALMGGIAALFGVPWLSAATVRTVTHCNALTVMSKSV  732
sAE2    TLIISKKERMLTKGSGFHLDLLIIVGSGGVAAMFGLPWLAAATVRTVTHVNALTVMSKSV 1051
sAE3    TLIVSKKERHLVKGSGFHLDLLLIGVGGGFCGIFGLPWQAAATVRSIAHVNALTVMSNST 1053
        ***:** :* :.**********:*   **...:**:** :*****:::* *******:*.

sAE1    PPGHKPQIQEVKEQRITGFIVAILIGLSILIGNILRNIPLAVLFGIFLYMGVTSLNGIQL  792
sAE2    APGDKPKIEEVKEQRVTGLVVAIMVGLSIVIGDLLRKIPLAVLFGIFLYMGVTSLNGIDL 1111
sAE3    APGEKFRIQEVKEQRVTGIVVAVLVGLSVVMSQVLRMIPMAVLFGIFLYMGVTSLTGIQI 1113
        .**.* :*:******:**::**:::***:::.::** **:***************.**::

sAE1    FDRLLLLFIPPKYHPDLSYVRKVHTRRMHIFTVIQLLCIVILWAINKSAFSLAFPFVLIL  852
sAE2    YERIQLLLMPSKHHPDHMYVRKVRTLRMHLYTIVQVFCLAVLWAVMSTVASLAFPFVLIL 1171
sAE3    YERLLLMFVPSKLHPDHVYVTKVRTWKMNMFTCIQLFCITILWIIKSTIISLAFPFFLIL 1173
        ::*: *:::*.* ***  ** **:* :*:::* :*::*:.:** : .:  ******.***

sAE1    TVPLRMFALGKIFSPVEMKSLDGDEVEATFDEQAGKDVYDEIPMPS  898
sAE2    TVPVKMFALTRIFTDRELKCLDADDAQPTFDEKEGLDEYDELSMPV 1217
sAE3    TVPTRRYLLPKIFHERELMALDSDEIHPNFDED-GHDEYSEMHMPQ 1218
        *** : : * :**   *: .**.*: ...***. * * *.*: **
```

Fig. 2. Alignment of the three skAEs. The alignment was done using ClustalW comparing the cytoplasmic domains of the exchangers and then the membrane-spanning domains. Lines above sequences show putative membrane-spanning domains of the proteins. Bold letters are putative glycosylation sites. Stars under sequences point out identical amino acids between the three skAEs. Single dots and double dots point out one or two difference(s) in nucleotidic sequence, respectively. Accession numbers: AJ537571 for skAE1, AJ537572 for skAE2 and AJ537573 for skAE3.

hAE2. The membrane-spanning domain of skAE2 is highly conserved (87.5 and 89% similarity with hAE2 and cAE2, respectively) with major differencies in the Z-loop. This loop has three putative glycosylation sites as in the other known AE2.

skAE3 shares 73.4% of similarities with hAE3 and around 70% with rabbit AE3. These similarities increase when we compare only the membrane-spanning domains (up to 80.3% similarity), and it is 60% in the hydrophylic cytoplasmic domain. A noticeable difference between skAE3 and mammalian AE3 is the size of the extracellular loop connecting putative helices 5 and 6, which is shorter in skAE3 (14 amino acids less), having about the same length as in skAE1 (Fig. 2). This loop carries the only putative glycosylation site of skAE3. A strong cluster of acid residues found in the N-terminal part of all known mammalian AE3 is conserved in skAE3 (aa 136–157).

The carbonic anhydrase II binding site identified in hAE1 has the acidic sequence D887ADD critical for binding (Vince, 2000). Similar acidic residues are found also in the three skAEs: this sequence is D874GDE in skAE1, D1193ADD in skAE2 and D1195SDE in skAE3.

Expression in *Xenopus* Oocytes

To investigate the anion exchange activity of these three skAE, they were expressed in *Xenopus* oocytes and ^{36}Cl uptake measurements were done. We determined the amount of cRNA for each isoform to be injected to obtain maximal expression in terms of Cl influx. Injection of *Xenopus* oocytes with optimal concentration of skAE1 or skAE2 cRNA resulted in a significant increase in ^{36}Cl uptake (Fig. 3). The Cl uptake in control oocytes was substracted from skAE1- or skAE2-induced Cl uptake. In skAE1-ex-

Table 1. Comparison of amino-acid sequences between the three skate AEs and human AEs

		hAE1			hAE2			hAE3		
		identity %	similarity %	length aa	identity %	similarity %	length aa	identity %	similarity %	length aa
Full length	skAE1	56.9	72.4	869						
	skAE2				64.9	77.7	1232	56.1	69.6	1237
	skAE3				55.8	69.6	1240	59.5	73.4	1205
Spanning domain	skAE1	69.4	82.9	510	65.6	79.8	536	62.5	77.7	526
(≈530 a.a.)	skAE2	67.9	82.3	528	76.8	87.5	540	66.9	81.1	542
	skAE3	60	77.5	521	64.5	77.9	539	66.7	80.3	530

The access numbers for hAEs sequences were NP_00333.1 (hAE1), NP_003031.2 (hAE2) and NP_005061.1 (hAE3). Comparisons are given in % of identity or similarity. The length refers to the number of amino acids that have been compared to obtain the %. For comparison over full-length proteins, skAE1 is only compared to hAE1 (911 amino acids), since AE1s are much shorter than the two other AEs (1240 amino acids for hAE2 and 1232 aa for hAE3), and a comparison of data would not have been possible.

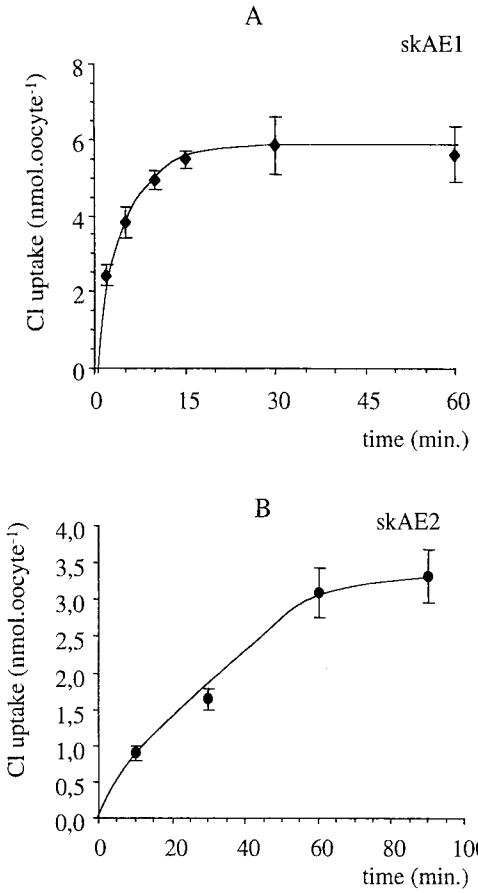

Fig. 3. Cl uptake by skAE1- (*A*) or by skAE2- (*B*) injected oocytes as a function of time. cRNA of skAE1 (from pGEMT-easy vector, 25 ng) or skAE2 (from pSP54polyA vector, 4 ng) were injected into defolliculated oocytes. ^{36}Cl uptake measurements were done three days after injection. For each experiment, each uptake was done on eight oocytes individually counted. Data presented are an average of three different experiments. (means ± SE., n = 3). The same results as (*A*) were obtained when 4 ng of skAE1 cRNA from pSP64polyA vector were injected into oocytes.

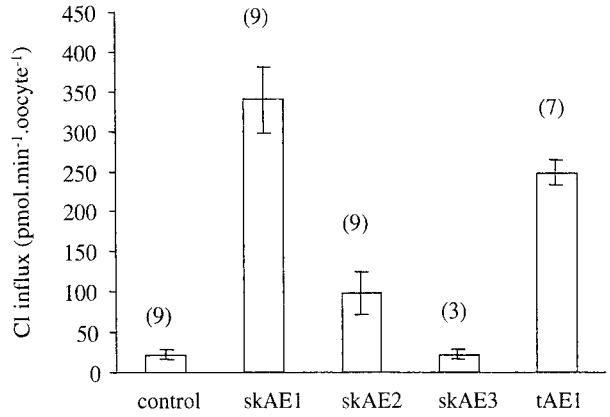

Fig. 4. Comparison of Cl influx of oocytes expressing different isoforms of AEs: skAE1, skAE2 and skAE3 compared to tAE1 (trout AE1) and non-injected oocytes (*control*). Cl influx was measured 3 days after injection, in 5 minutes for skAE1, in 15 minutes for tAE1 and in 30 minutes for skAE2 or skAE3. Results are expressed in pmol of Cl per min per oocyte and are a mean of different experiments (number in parentheses), each of them done with 8 oocytes individually counted and then averaged.

pressing oocytes, this uptake is linear for about 15 minutes and then reaches a plateau value. For skAE2 the uptake is linear for 60 minutes before reaching the plateau value. Subsequent experiments were done for periods of 5 minutes with skAE1 and of 30 minutes for skAE2, so that data presented correspond to initial rates of Cl influx. Figure 4 illustrates the Cl influx of skAE1-injected *Xenopus* oocytes compared to skAE2- and skAE3-injected oocytes. Only two isoforms of skAEs increased the Cl permeability of oocytes. skAE3 was not able to increase Cl transport in *Xenopus* oocyte. However, it was observed that this cDNA was efficiently translated in vitro by rabbit reticulocyte lysate. skAE1-induced Cl influx is about three times higher than skAE2-induced Cl influx and it is comparable to the Cl influx induced by tAE1 expression in *Xenopus* oocytes.

Cl uptake was also measured at different days following injection of skAE1 or skAE2 or skAE3. It was observed that 2 days after injection Cl influx is maximal for skAE1- and skAE2-expressing oocytes and it is then stable for at least 6 days (*data not shown*). skAE3 was not able to increase oocyte Cl permeability even six days after injection.

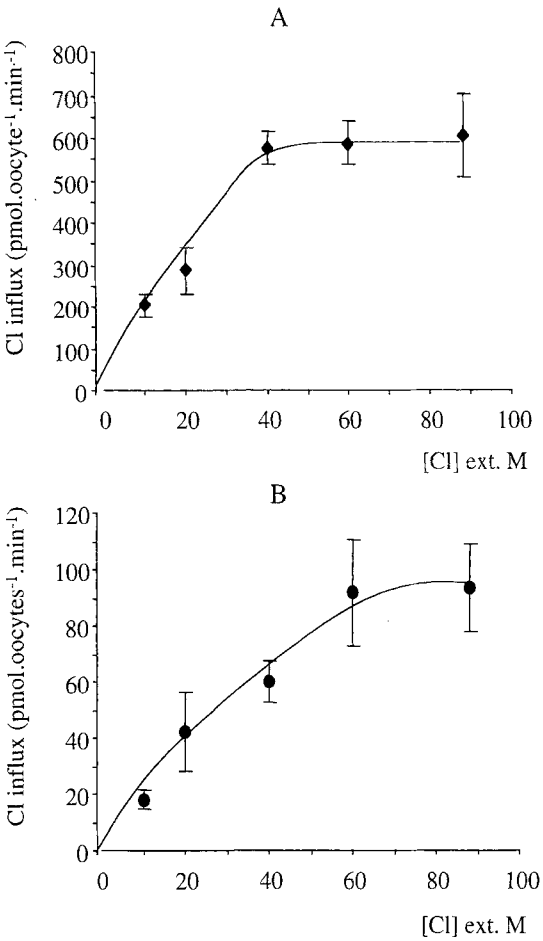

Fig. 5. Cl influx as a function of external Cl concentration in oocytes injected with skAE1 (*A*) or skAE2 (*B*). Influx was measured three days after injection. Cl uptake was measured during the linear period of the kinetics in MBS where NaCl was partly substituted by Nagluconate to be able to vary external Cl concentration. There were 5 different Cl concentrations: 10, 20, 40, 60 and 88 (regular MBS) mM. Data presented correspond to one representative experiment of 3, done with 8 individually counted oocytes. (mean ± SE, $n = 8$).

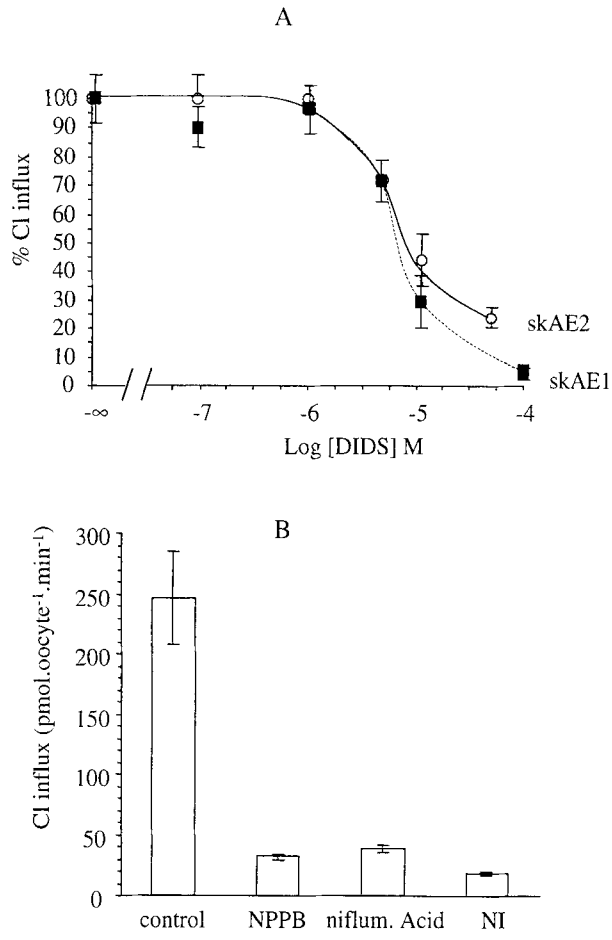

Fig. 6. (*A*) Dose-response curve of the inhibition by DIDS of Cl influx induced by skAE1 (*black squares*) or skAE2 (*white circles*) expression in *Xenopus* oocytes. A stock solution of DIDS was made in DMSO at 10^{-1} M then diluted in MBS to the desired final concentration. (Means ± SE., $n = 4$ for skAE1 and means ± SE., $n = 3$ for skAE2). (*B*) Cl influx in oocyte expressing skAE1 in the absence (*control*) or presence of different inhibitors: NPPB 5×10^{-4} M; Niflumic acid 5×10^{-4} M compared to non-injected oocytes (*NI*) (means ± SE., $n = 8$). Stock solutions of inhibitors were made at 10^{-1} M in DMSO.

The dependence of skAE1 or skAE2-associated ^{36}Cl influx on the concentration of external Cl is illustrated in Fig. 5. For both transporters half saturation is observed at about 25 mM of external Cl. Saturation is obtained at 40 mM external Cl for skAE1 but at 60 mM for skAE2. Sensitivity of skAE1 and skAE2 isoforms to DIDS was assessed. As illustrated in Fig. 6*A*, DIDS is a potent inhibitor of skAE1 and skAE2 expressed in *Xenopus* oocytes with an IC50 of 6 μM and 8 μM, respectively. Other inhibitors such as niflumic acid and NPPB also inhibit Cl influx in skAE1 injected oocytes (Fig. 6*B*).

Discussion

The data presented here suggest for the first time the presence of three AE isoforms in a red cell. Only two of these three isoforms of skAE are able to induce Cl uptake when expressed in *Xenopus* oocytes. Moreover, skAE1-induced Cl influx is higher than skAE2-induced Cl influx, despite injection of saturating concentrations of cRNA in oocyte. The Cl influx measurement is correlated to the number of exchangers in oocyte membrane and to the transport efficiency of the protein. Therefore, we can not conclude from Cl influx experiments the relative efficiency of the transporters in carrying anions through oocyte plasma membrane, because we do not know how much of each protein is present in this membrane. However, these differences in Cl influx intensity induced by skAE1, skAE2 or skAE3 indicates that these transporters are not identical with each other regarding their expression characteristics in the oocyte.

It is possible to compare each of these transporters to other corresponding members of the AE family studied by expression in *Xenopus* oocyte.

The Cl influx induced by skAE1 is comparable to that observed by expression in *Xenopus* oocytes of other AE1 isoforms derived from different species such as trout (Fievet et al., 1995) or mouse AE1 (Chernova et al., 1997). Cl influx induced by skAE2 is quantitatively comparable to what was observed with murine AE2 isoform (Humphreys et al., 1994). In contrast to what was observed with human brain and cardiac AE3 (Yannoukakos et al., 1994), we were not able to functionally express skAE3 in *Xenopus* oocytes. The cloning vector we used, pSP64polyA, may be poorly adapted to expression of this isoform in oocyte. Thus, further expression studies need to be done to conclude about the functionality of skAE3. However, it should be mentioned that the Cl uptake mediated by rat AE3 in *Xenopus* oocyte is very low and most of the studies to characterize mammalian AE3 were done on transfected cultured cells like HEK293 by measuring variations in intracellular pH in response to modifications of external Cl concentration (Lee, Gunn & Kopito, 1991; Sterling & Casey, 1999; Alvarez, Fujinaga & Casey, 2001). This suggests that the *Xenopus* oocyte is probably not the most suitable expression system to study skAE3 and this isoform could, like the other members of the AE3 family, not be so efficient in Cl exchange as skAE1 or skAE2 (Sterling & Casey, 1999). Thus, these three new AE isoforms share expression features in oocyte in common with the other corresponding members of AE family that have been studied.

The Cl dependence of skAE1-mediated Cl uptake is 25 μM, as for skAE2. It is similar to what was observed with tAE1 expressed in *Xenopus* oocytes (H. Guizouarn, unpublished data). However, this K_m is higher than what was observed with mammalian AEs : 5.6 μM for mouse AE2 (Humphreys et al., 1994) and 8.3 mM for mouse AE1 (Passow, 1992) expressed in *Xenopus* oocyte. There is a similar K_m between AE1 and AE2 coming from mammalian erythrocytes and kidney, just as there is a similar K_m between skAE1 and skAE2 coming from skate erythrocytes, suggesting that, in a given animal, the two isoforms AE1 and AE2 may be functionally close to each other despite different tissue origins.

This functional relationship is confirmed by the DIDS sensitivity of skAE1 and skAE2. Expressed in *Xenopus* oocytes, both isoforms have nearly the same sensitivity to this inhibitor, with an IC50 of 6 and 8 μM respectively. The mouse AE2 is half inhibited by 13 μM of DIDS in *Xenopus* oocyte (Humphreys et al., 1994), but half-maximal inhibition is obtained with 142 μM of DIDS when this exchanger is expressed in HEK 293 (Lee et al., 1991). It appears that DIDS sensitivity could vary for the same isoform, depending on the expression system used and on temperature or ionic conditions. DIDS sensitivity of AEs expressed in *Xenopus* oocyte may not be representative of what it is in the physiological environment. In agreement with this suggestion, it should be pointed out that tAE1, which is sensitive to DIDS in trout erythrocyte (a plasma membrane that is rich in cholesterol), is insensitive to this compound once expressed in *Xenopus* oocyte. The DIDS sensitivity of this exchanger in *Xenopus* oocyte was restored by shortening the length of the extracellular loop connecting membrane spans 5 and 6 (Fievet et al., 1995). Obviously, the native conformation of the exchanger is different in the two cell types. However, focusing on the same expression system, the *Xenopus* oocyte, AE1 and AE2 have about the same sensitivity to DIDS whether they come from the mouse or skate species. This confirms some functional relationship between AE1 and AE2 isoforms.

To conclude about functional characteristics of skAE expressed in *Xenopus* oocyte, these transporters have an expression pattern close to other known corresponding AEs. Despite an obvious difference in the expression level of the three isoforms in oocyte, skAE1 and skAE2 share some functional similarities (Cl dependence and DIDS sensitivity), as was observed also for other AE1 and AE2 derived from different species.

Many putative glycosylation sites are found in different extracellular loops of the three skAE. skAE1 has three putative N-glycosylation sites in the extracellular loop, connecting spans 5 and 6, and one in the next extracellular loop between spans 7 and 8. Among the three putative glycosylation sites in the first loop, only the first two should be glycosylated (Landolt-Marticorena & Reithmeier, 1994): the asparagines 542 and 546, for instance, which are about 20 residues distant from either side of the lipid bilayer. The third asparagine in this loop (N554) is too close to the lipid bilayer. By comparison, in trout AE1 the two putative N-glycosylation sites found in the corresponding loop are a distance of 21 residues and are both glycosylated (Borgese et al., manuscript in preparation). The putative glycosylation site (N635) in the next extracellular loop of skAE1 is present and glycosylated in hAE1 (Casey, Pirraglia & Reithmeier, 1992; Landolt-Marticorena & Reithmeier, 1994). Landolt-Marticorena and Reithmeier (1994) found it unlikely that two adjacent extracellular loops could be glycosylated in multi-span membrane glycoproteins. However, using an N-glycosylation mutant of hAE1 it was found that this protein could be glycosylated on multiple extracytosolic loops (Tam L.Y., 1996). Thus, skate AE1 could either be glycosylated like tAE1, on the first extracellular loop carrying glycosylation sites, or like hAE1, on the second extracellular loop carrying a glycosylation site, or on both loops. This isoform is remarkable in regards to its putative glycosylations

compared to other known AEs; this particularity would be worth further studies.

skAE2 has the same putative glycosylation sites as skAE1 in the loop connecting spans 5 and 6. These three sites are also found in AE2 from other species and they are glycosylated (Lindsey et al., 1990). In case of mammalian AE2, there are at least 10 residues between each site; that is not the case for skAE2. If the short distance between the first two asparagines (N845 and N849) in skAE2 does not prevent N-glycosydase action on both sites, this isoform may be glycosylated like other known AE2s. skAE3 could be glycosylated on N865 in the loop connecting helices 5 and 6, like rat AE3 (Kobayashi et al., 1994).

The presence of RNA coding for three AE isoforms in skate red cells suggests that the three corresponding proteins are expressed in these cells. Coomassie blue staining of electrophoresis separation of skate erythrocyte membrane proteins failed to show any strong band around 100 kDa corresponding to a putative anion exchanger. Two faint bands, one around 100 kDa and the other one around 160 kDa are visible. The lower band is recognized by an antibody against human AE1, suggesting that skAE1 could be this 100 kDa band (Musch et al., 1994). Moreover, this band is also labelled by $[^3H]H_2DIDS$. Wether the 160 kDa band corresponds to skAE2 and skAE3 has to be assessed by the use of specific antibodies raised against each isoform.

The possible presence of three different AE isoforms in skate erythrocytes raises the question about their respective functions in these cells. In erythrocytes, the main function of the anion exchanger is to increase CO_2 transport. The CO_2 is hydrated in red cells, giving HCO_3^-, which is then exchanged via the anion exchanger for extracellular Cl^-. Cardiac myocytes, vascular smooth muscle cells and intestinal cells are the only other known examples of cells where the presence of different isoforms of AEs has been reported. This was interpreted as allowing a better control of cellular pH homeostasis (Brosius et al., 1997; Sterling & Casey, 1999). Some isoforms, like AE2, have their optimal transport capacities at alkaline pH. In contrast, AE1 is insensitive to changes in pH (Humphreys et al., 1994; Sekler, Kobayashi & Kopito, 1996). The simultaneous presence of these isoforms allows the stability of cellular pH over a wide range of pH alteration. Such pH-regulation function for AEs in red cells does not seem relevant since these cells are not able to regulate their pH. They follow the Donnan equilibrium: there is a constant ratio between intracellular and extracellular H^+ concentrations. It remains possible, however, that these isoforms are involved in red cell volume regulation. In trout erythrocyte, the anion exchanger tAE1 was shown to be involved in taurine and cation transport in response to cell swelling with a decrease in intracellular ionic strength (Garcia-Romeu et al., 1991; Guizouarn & Motais, 1999). In skate erythrocytes, pharmacological and biochemical evidences suggest the involvement of AEs in the swelling-induced taurine permeability (Goldstein & Brill, 1991; Musch et al., 1994). In these cells, antibodies raised against hAE1 were able to immunoprecipitate an anion exchanger that was shown to be oligomerized by hyposmotic swelling. This structural modification was correlated with an increase in taurine permeability of these cells (Musch et al., 1999). The similarities between hAE1 and skAE1 sequences suggest that skAE1 could be the isoform immunoprecipitated in skate red cell and involved in swelling-induced taurine permeability. However, these similarities between skate AE1 and human AE1 do not favor the possibility that skAE1 is able to form a channel permeable to taurine as tAE1, since neither human AE1 nor mouse AE1 are able to transport taurine. Another hypothesis is that skAE1 forms functional oligomeres able to transport taurine with skAE2 and/or skAE3. Studies on the involvement of these skAEs in taurine uptake are subject of ongoing experiments.

The knowledge of three new sequences of AEs coming from a fish phylogenetically more ancient than trout will contribute to the understanding of the specific functions of these anion exchangers. Especially the presence of these three isoforms in a nucleated red cell raises many questions still unresolved. Answers to these questions could help to understand the possible functional relationship between the exchangers.

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