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Proton-sensitivity of ASIC1 appeared with the rise of fishes by changes of residues in the region that follows TM1 in the ectodomain of the channel

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Running Title: ASIC1 from early vertebrates is not gated by protons.

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ABSTRACT

The acid-sensitive-ion channel 1 (ASIC1) is a neuronal sodium channel insensitive to changes in membrane potential but is gated by external protons. Proton-sensitivity is believed to be essential for the role of ASIC1 in modulating synaptic transmission and nociception in the mammalian nervous system. To examine the structural determinants that confer proton sensitivity we cloned and functionally characterized ASIC1 from different species of the chordate lineage: lamprey, shark, toadfish and chicken. We observed that ASIC1s from early vertebrates (lamprey and shark) were proton-insensitive in spite of a high degree of amino acid conservation (66 to 67%) with their mammalian counterparts. Sequence analysis showed that proton-sensitive ASIC1s could not be distinguished from proton-insensitive channels by any signature in the protein sequence. Chimeras made with rat ASIC1 (rASIC1) and lamprey or shark indicated that most of the ectodomain of rASIC1 was required to confer proton-sensitivity and the distinct kinetics of activation and desensitization of the rat channel. Proton-sensitive chimeras contained the segment D_{78}-E_{136} together with residues D_{351}, Q_{358} and E_{359} of the rat sequence. However, none of the functional chimeras containing only part of the rat extracellular domain retained the kinetics of activation and desensitization of rASIC1 suggesting that residues distributed in several regions of the ectodomain contribute to allosteric changes underlying activation and desensitization. The results also demonstrate that gating by protons is not a feature common to all ASIC1 channels. Proton sensitivity arose recently in evolution implying that agonists different from protons activate ASIC1 in lower vertebrates.

KEY WORDS: Shark, fish, chicken, lamprey, ASIC1, proton sensitivity.

INTRODUCTION

The ASICs belong to the large class of ENaC/Degenerin channels, which is distinguished by the presence of two transmembrane domains and a single large extracellular loop. Human and rat ASIC2a were the first cloned member of this family (Price *et al.* 1996; Waldmann *et al.* 1996); since then, four genes and six spliced forms have been identified in mammals. Later, Waldmann et al. observed that external protons activated these channels (Waldmann *et al.* 1997) providing the molecular identity of the proteins that carry acid-activated currents in sensory neurons first reported by Krishtal more than two decades ago (Krishtal & Pidoplichko, 1981) and later found in most neurons of the mammalian brain.

Not all of the functional roles of ASICs have been definitively established but several putative functions have been proposed. Expression in nociceptors and activation by protons suggest that the ASICs may act as pain receptors (Chen *et al.* 1998; Immke & McCleskey, 2001), mainly because ASIC1 and ASIC3 exhibit the highest proton affinity among the ASICs. In the central nervous system ASIC1 is the most abundant and most ubiquitously expressed of all the ASICs. ASIC1 has been implicated in modulating synaptic transmission, memory and fear conditioning (Wemmie *et al.* 2002; Wemmie *et al.* 2003). Most recently, it has been shown that ASIC1 mediates cell injury induced by ischemia, inflammation and other conditions associated with acidosis in the mammalian nervous system (Xiong *et al.* 2004). Although the precise mechanisms underlying these effects have not been elucidated, the prevaling notion is that protons released by synaptic vesicles activate ASIC1 enhancing depolarization of post-synaptic membranes.

The goals of this work were first, to define structural determinants in ASIC1 that render the channel sensitive to protons, and second, to investigate whether proton-sensitivity is

conserved in the chordate lineage. For that purpose we cloned and characterized ASIC1 from lamprey (*Lampetra fluviatilis*) as representative of cyclostomes, the dogfish (*Squalus acanthias*) from chondrichthyes, additional channels from the teleost *Opsanus tau*, and from chicken (*Gallus gallus*). We report the sequences and results of functional studies of the recombinant proteins expressed in *Xenopus* oocytes and of chimeras made with shark or lamprey and rat ASIC1. The data indicate that proton-sensitivity is not a universal feature of ASIC1. In the evolution of the chordate lineage proton-sensitivity was acquired with the rise of fishes. The determinants of proton sensitivity are located in noncontiguous regions of the ectodomain and are tightly linked to the kinetics of activation and desensitization, indicating that an allosteric mechanism underlies gating by protons.

MATERIALS AND METHODS

Cloning of shark, fish, lamprey and chicken ASIC cDNAs. Prior to removal of brain or spinal cord from shark, toadfish and lamprey, the animals were anesthetized with 0.5% tricaine added to the water. Chicken was first anesthetized by ether inhalation followed by decapitation. Total RNA was extracted using the TRIZOL reagent (Invitrogen). First strand cDNA synthesis was conducted with 5 μg of total RNA using oligodT primers and SuperScript RT II reverse transcriptase (Invitrogen). Screening for ASIC message was performed by PCR using degenerate primers from highly conserved sequences corresponding to NCNCRMVHMPG (sense: AA(C/T)TG(C/T)AG(A/G)ATGGTICA(C/T)ATGCCIGG) and GDIGGQMG (reverse: CCCAT(C/T)TGICCICCIAT(A/G)TCICC). I stands for inosine. PCR was performed with Taq polymerase (Invitrogen) with the following parameters: 20 s denaturing at 94° C, 30 s annealing at 55° C, and 30 s extension at 72° C, repeated for 30 cycles. PCR products were sequenced and used to design specific primers for 5' and 3' RACE (Rapid Amplification of cDNA Ends). Whole-length cDNAs were obtained using the GeneRacer System for full length, RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (Invitrogen). Lamprey ASIC1 cDNA was tagged with FLAG epitope at the carboxy-terminus and subcloned in pCRIITOPO vector (Invitrogen). Shark ASIC1α and ASIC1β cDNAs were subcloned in pcDNA3.1V5/HisTOPO vector (Invitrogen), which provides the V5 epitope at the carboxy-terminus of the protein.

To search for other ASIC genes different from toadfish ASIC1 (fASIC1), we treated the 360 bp PCR fragment obtained with the above pair of degenerated primers with DNA restriction enzymes: StuI, StyI or MluI (sites present in fASIC1). After digestion, the DNA was amplified again by PCR using the same degenerated primers. Template digested with StuI or

StyI did not yield reamplification products in contrast to template digested with MluI. DNA sequence of products digested with MluI identified two additional fASICs. Based on the new sequences, we designed specific primers for 5'RACE and 3'RACE to amplify the complete coding region of the cDNAs. We introduced a FLAG epitope in the reverse primer. The PCR products were subcloned in pCRII TOPO Dual Promoter Vector (Invitrogen).

Chicken ASIC1 was cloned by RT-PCR using the following sense and reverse primers: C G G A C C A T G A T G G A C C T G A A G G T G G A C G A G G and GCAGGTGAAGTCCTCAAAGGTGCCCCGG located in the 5' and 3' of the predicted coding sequence of ASIC1 (NCBI accession No XM-424489). DNA of at least three independent clones of each of the new ASIC reported here were sequenced with an automatic sequencer at the Keck Facility at Yale University.

Construction of chimeras and site directed mutagenesis. Chimeras were made with rat and lamprey ASIC1 cDNAs and with rat and shark ASIC1α. To make lamprey-rat CH1 we introduced a *HindIII* site at amino acid position 78 and a *StuI* site at position 424 of lamprey ASIC1 protein. Lamprey cDNA was digested with *HindIII* and *StuI* and ligated with the corresponding *HindIII* and *StuI* fragment from rat ASIC1 cDNA. For chimeras CH3 and CH4 *XbaI* and *NheI* sites were introduced in positions 187 and 346 of lamprey and the corresponding site in the rat protein. The sites were returned to the original sequences by a second round of site directed mutagenesis using Quickchange (Stratagene). For CH5, CH6 and CH7, *SacII* sites were introduced at positions 150, 136 and 128 in lamprey and rat. The restriction sites *SacII* did not change the sequence of the proteins. To make shark-rat CH1 we

introduced *HindIII* and StuI sites in the shark ASIC1α cDNA at positions corresponding to 78 and 424, and for CH2, *SacII* and *NheI* sites at positions corresponding to 150 and 346.

Western blotting and surface biotinylation of oocytes. After cRNA injection oocytes were incubated at 18° C for three days prior to experiments. 20 oocytes from each group were washed with ice-cold buffer A (mM): 90 NaCl, 3 KCl, 1 $CaCl_2$, 5 Triethanolamine, pH 8.0, and then transferred to 200 μl of biotinylation solution, buffer A containing 1.5 mg/ml of freshly prepared sulfo-NHS-SS-Biotin (Pierce, Rockford, IL). Cells were incubated on a rocker at 4° C for 20 min. Fresh biotinylation solution was added a second time after which cells were transferred to a 5 ml container with ice-cold quenching solution (buffer A with 50 mM glycine) and incubated for 10 min. Oocytes were transferred to a microfuge tube containing 400 μl of buffer B (in mM): 90 NaCl, 20 Tris, pH 7.4, 1% TritonX-100. Homogenates were cleared by centrifugation (10,000 rpm) for 10 min. The supernatant was mixed with 50 μl of streptavidin bead slurry (Pierce) and incubated on a rocker overnight at 4° C. Beads were separated by centrifugation and washed three times with buffer B and three times with buffer B supplemented with 300 mM NaCl. 50 μl of loading buffer containing DTT were added to the beads and heated at 90° C for 5 min. The eluted proteins were loaded on 10% SDS-PAGE together with 10 μl of supernatant recovered after incubation of lysate with streptavidin beads. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked with 5% non-fat dry milk in TBST (in mM: 20 Tris, pH 7.6, 120 NaCl, 0.1% Tween) for 30 min, then incubated for 2h at room temperature with anti-*FLAG* or V5 monoclonal antibodies at a dilution 1:5000. After three 15-min washes with TBST, they were incubated for 1h at RT in a 1:10000 dilution of horseradish peroxidase

labeled anti-mouse secondary antibody (Sigma). Membranes were washed three times with TBST, signals were developed with ECL$^+$ (Amersham Pharmacia Biotech) according to the manufacturer's protocol and exposed to BioMax MR film (Eastman Kodak, New Haven CT).

Co-immunoprecipitation. After electrophysiological measurements, oocytes were homogenized in non-denaturing lysis buffer (in mM): 50 Tris pH7.4, 150 NaCl, 1 EDTA, 1%TX-100, and COMPLETE protease inhibitors (Roche), cleared by centrifugation at 12,000 rpm for 10 min in a microcentrifuge followed by 10-fold dilution with homogenization buffer and incubation with 5 μl of anti-HA polyclonal antibody (Convance, Inc.) for 3 h at 4^o C. Immune complexes were recovered with protein A linked to sepharose beads (Sigma). After several washes with homogenization buffer, complexes were eluted from the beads with Laemmli loading buffer containing β-mercaptoethanol and resolved in 7.5% SDS-PAGE. Proteins were transferred to Immobilon membranes (Millipore) and were blotted with monoclonal anti-FLAG (Sigma).

Expression in *Xenopus* oocytes and Two-electrode voltage clamp (TEVC). Stage V and VI oocytes were harvested from female *Xenopus laevis*, which were anesthetized using 0.17% tricaine prior to the operation. Oocytes were injected with 4 ng of single cRNA or combinations of cRNAs as indicated in the experiments. Plasmids containing the coding sequence of the ASIC genes were linearized at the 3' end to serve as template for synthesis of capped cRNAs with T7 polymerase, mMessagemMachine kit (Ambion, Austin, TX). Injected oocytes were examined with the TEVC after 2 to 4 days. Oocytes were placed in a recording chamber (400 μl) perfused by gravity at a rate of 2-3 ml/min. Oocytes were

impaled with two glass microelectrodes filled with 3 M KCl having resistance lower than 1 MΩ. Whole-cell currents were recorded with a Clamp OC-725B (Warner Instrument Corp., Hamden, CT), digitized at a sampling rate of 2 kHz (PowerLab/200, ADInstruments) and the data stored in a computer. Composition of the standard bath solution was (mM): 150 NaCl, 2 KCl, 5 $CaCl_2$, 10 HEPES-MES adjusted to pH 7.4. Activation solutions were administered by a perfusion system positioned directly in front of the oocyte. Composition of activating solutions was (mM): 150 NaCl, 2 KCl, 1 $CaCl_2$, 10 HEPES-MES or 10 MES adjusted to pH 7.0 to 4.0.

Sequence analysis of ASIC1. ClustalX program (Thompson *et al.*, 1997) was used to align and calculate conservation score (c-score) of the protein sequences of human, rat, chicken, zebrafish, toadfish, shark and lamprey ASIC1. C-scores were computed for each position in the multiple sequence alignment, separately for proton- sensitive and -insensitive groups. These scores were then averaged over a sliding window of seven amino acids with a step of four amino acids and plotted on y-axis and residue position on x-axis. Differences in evolutionary rate between the two ASIC1 groups were inferred by computing the ratio between the nonsynonymous versus synonymous rates of substitution (Ka/Ks) using the YN00 program within the PAML evolutionary package (Nei & Gojobori, 1986; Yang 1997). Analysis was performed on cDNA sequences using a window of 90 nucleotides (30 codons) with a step of 12 nt.

RESULTS

Cloning and functional expression of chicken ASIC1

Chicken ASIC1 was cloned and characterized to determine the degree of protein conservation and to compare functional properties with another tetrapode different from the mammalian one. The sequence obtained from chicken ASIC1 (supplementary data) differed from the predicted by automated computational analysis (NCBI accession No XM-424489). The protein shares 89% amino acid identity with rat, mouse and human ASIC1. Functional expression in oocytes indicated that chicken ASIC1 is proton sensitive (calculated apparent EC_{50} for activation by external protons, pH 5.8) but it generates channels with rapid kinetics of activation and desensitization very different from the mammalian channels but indistinguishable from fish ASIC1 (data not shown). This is an unexpected finding given that the human and chicken proteins are more similar than chicken is to fish (89% vs. 77% identity). The rapid kinetics could be attributed to presence of residues $T_{84}R_{85}$ in chicken vs. $S_{83}Q_{84}$ in rat, which we had previously identified as important in determining the kinetics of desensitization (Coric *et al*. 2003).

Cloning of ASIC1.2 and ASIC2 channels from toadfish. We had previously cloned and characterized ASIC1.1 from toadfish (Coric *et al*. 2003). Here we searched for additional ASIC1 genes using the approach described in methods. We cloned two additional closely related cDNAs, one clone showed high sequence homology to mammalian ASIC1 and was labeled fASIC1.2; it is a paralog of fASIC1.1; whereas the other sequence was most closely related to mammalian ASIC2. This clone was labeled fASIC2. The three proteins exhibit 73-78% conservation and 51-54% overall amino acid identity. The regions of highest homology

are M2 and the ectodomain whereas the cytoplasmic amino- and carboxytermini as well as M1 are less conserved.

Relation of the three toadfish proteins with all the other ASICs cloned to date is shown in the dendrogram in Fig. 1. The degree of amino acid identity of fish and rat ASICs is 75% for fASIC1.1, 61% for fASIC1.2, and 56% for fASIC2.

Functional characterization of fish ASIC1.2 and ASIC2 in *Xenopus* oocytes. To examine the functional properties of fASIC1.2 and fASIC2 we injected oocytes with cRNAs of these clones alone or in combination with toadfish ASIC1.1. Upper races in Fig. 2A show that fASIC1.1 induces currents that activate and inactivate rapidly as we previously described (Coric *et al.* 2003). In contrast, oocytes injected with fASIC1.2 or fASIC2 did not exhibit proton-sensitive channels. Co-injection of fASIC1.1 with the other fish clones produced currents with properties similar to those of fASIC1.1 but of smaller magnitude whereas co-injection of fASIC1.2 with fASIC2 did not generate proton-activated currents (lower traces in Fig. 2A). A summary of the data from two independent experiments normalized to the values obtained with fASIC1.1 is shown in Fig. 2B. All the conditions exhibited statistically significant smaller currents than fASIC1.1 alone ($p \leq 0.001$).

Mutations of residue G430 located in the outer pore of the mammalian ASIC2a render the channel constitutively active and more sensitive to external protons (Huang & Chalfie, 1994; Champigny *et al.* 1998). We thus substituted the corresponding amino acids in fASIC1.2 (G458F) and fASIC2 (G431F); however, these mutations did not induce gain of function whether the mutant channels were expressed alone or in combination with fASIC1.1.

Fish ASICs form heteromeric complexes. The inability to induce proton-gated currents was not due to the absence of protein expression because western blots revealed abundant expression of all three subunits. Fig. 3A shows fASIC1.1 detected with anti-HA and fASIC1.2 and fASIC2 with anti-FLAG antibodies. fASIC1.2$_{G458F}$ and fASIC2$_{G431F}$ mutants were also expressed in oocytes that did not exhibit proton-gated currents (right panel, Fig. 3A). Moreover, using surface biotinylation we demonstrated that fASIC1.2 and fASIC2 reach the plasma membrane (Fig. 3B).

The decrease in fASIC1.1 current induced by co-expression with the other fish ASICs raised the possibility that fASIC1.1 associates with ASIC1.2 and/or ASIC2 to form heteromeric channels that are insensitive to external protons. We therefore conducted co-immunoprecipitations from oocytes expressing fASIC1.1+1.2 or fASIC1.1+2. Non-denaturing immunoprecipitations were performed with rabbit anti-HA antibody that recognizes fASIC1.1 followed by detection of fASIC1.2 and fASIC2 with anti-FLAG monoclonal antibody by western blotting. The western blot in Fig. 3C shows the bands corresponding to ASIC1.2 and ASIC2, indicating assembly with fASIC1.1. Therefore, reduction of the magnitude of fASIC1.1 proton-induced currents by co-expression with ASIC1.2 and ASIC2 most likely reflects the formation of non proton-gated heteromeric channels.

Expression of lamprey and shark ASICs in *Xenopus* oocytes. We cloned two cDNAs from shark brain and one from lamprey spinal cord. The shark proteins differed only in the first aminoterminal third (intracellular aminoterminus, TM1 and first segment of the extracellular domain) but were identical in the distal two-thirds. The site of identity starts in the same

position as the rat ASIC1 and ASIC2 spliced forms. Comparison with other sequences in Gene Bank indicated that the two shark clones share closest identity with ASIC1, 66% amino acid identity with rat ASIC1; therefore, these cDNAs correspond to two splice forms of the shark ASIC1 gene: sASIC1α and sASIC1β.

The cDNA from lamprey also corresponded to ASIC1; it shared highest identity (66.7%) with rat ASIC1. Alignment of sASIC1α, sASIC1β and lASIC1 is shown in supplementary data and their relation with other ASICs is shown in the dendrogram of Fig. 1.

Functional properties of lamprey and shark ASIC1 channels were examined in *Xenopus* oocytes by the TEVC technique. Oocytes expressing single cRNAs of lASIC1, sASIC1α, or sASIC1β or co-expressing sASIC1α and sASIC1β did not respond to a rapid change of pH in the bathing solution (from 7.4 to 5.0). Additional maneuvers reported in the literature to increase or modify the response to protons were also tested: increase in Ca^{2+} concentration in the preconditioning solution to 6 mM (Babini *et al*. 2002; Coric *et al*. 2003), removal of Ca^{2+} from the low pH solution (0 mM) (Immke & McCleskey, 2004), pretreatment of oocytes with 200 μg/ml of trypsin (Poirot *et al*. 2004), and introduction of the degenerin's gain-of-function mutation, G410F (Champigny *et al*. 1998). However, none of these interventions rendered the channels sensitive to protons.

Only when lamprey or shark ASIC1 was co-expressed with rat ASIC1 did we detect proton-induced currents, but the magnitude of the currents was smaller than in oocytes expressing rASIC1 alone. Fig. 4A shows the results of injections with rat and shark cRNAs. Most of the oocytes injected with a rat:shark cRNA ratio of 1:10 expressed very small currents upon stimulation by protons (<0.3 μA/oocyte). When the ratio rat:shark was changed to 10:1, the average proton-induced current was 3.1±0.5 μA/oocyte, which represents 50% of the current

expressed by oocytes injected with the same amount of only rat ASIC1 cRNA. Injection of rat:lamprey cRNAs in ratios 1:10 and 10:1 produced similar results; although, the combination rat:lamprey in the ratio 10:1 expressed an average current of 1.3±0.6 μA/oocyte, which represents 25% of the current expressed by oocytes injected with rat ASIC1 alone (Fig. 4B). Thus, shark and lamprey ASIC1 channels seem to assembly with rASIC1 but the heteromeric channels are not proton-sensitive.

Because the absence of proton-induced currents with shark or lamprey ASIC1 may be due to the inability of assembly or delivery to the plasma membrane, these possibilities were examined by surface biotinylation of oocytes expressing shark or lamprey ASIC1s tagged in the carboxyterminus with the FLAG or V5 epitopes, respectively. Western blots in Fig. 5 show the presence of shark and lamprey ASIC1 in whole-cell lysates and in the plasma membrane. Therefore, homomeric lamprey and shark ASIC1 reach the plasma membrane but do not conduct upon stimulation by protons.

Proton-sensitivity of rat-lamprey chimeric channels. The difference in response to protons while maintaining a high degree of amino acid identity prompted us to generate chimeras between lamprey and rat ASIC1 in order to identify sequences important for conferring proton sensitivity to the channels. An alignment of the amino acid sequences of rat (top) and lamprey (bottom) ASIC1 is shown in Fig. 6A. Red and black indicate identical and different residues (numbers correspond to the positions indicated in Fig. 6B) and the boxes enclose TM1 and TM2. The sequence comparison makes evident a high degree of identity of TM2 and the distal two-thirds of the extracellular domain whereas the intracellular amino and carboxitermini and the first part of the extracellular domain are less conserved. Fig. 6B

shows a schematic representation of the chimeric constructs that were tested in oocytes. Represented in black are sequences from rat and in white sequences from lamprey; the numbers indicate the amino acid position in the rat sequence. Response with a transient inward current to a test pulse of pH_o 4.0 is indicated on the right column.

The first two chimeras, CH1 and CH2, have the whole extracellular domain exchanged between rat and lamprey while maintaining TM1, TM2 and the amino and carboxytermini from the parent protein. CH1 was not functional but CH2 exhibited properties indistinguishable from those of the rat ASIC1: EC_{50} for proton activation of pH_o 6.6, activation rate of 2 s^{-1}, and desensitization rate of 0.58 s^{-1} (Fig. 6C). To prove that CH1 was expressed at the plasma membrane by surface biotinylation. All the biotinylated proteins (Membrane) and 1/10 of the cytosolic fraction (Total) were revealed with anti-FLAG monoclonal (Fig. 6D). This result indicates that the extracellular domain of rat contains the elements that confer proton-sensitivity and also determines the kinetics of activation and desensitization.

Other chimeras were made to replace smaller segments of the rat extracellular domain. CH3 but not CH4 responded to pH_o 4.0; however, the currents of CH3 differed from rat ASIC1 and CH2 by having very rapid rise and decay rates, 10 s^{-1} and 2.9 s^{-1} and by a smaller magnitude of response, mean peak current of 2.2±0.4 μA/oocyte vs. 5.3±0.8 μA/oocyte. CH5 and CH6 exhibited properties indistinguishable from CH3 whereas CH7 was not functional suggesting that the short segment of amino acids comprised between positions 128 and 136 may be essential. To test this latter idea we generated CH8, which contains the segment 128-136 from rat while the other sequences were replaced by lamprey. CH8 did not respond to

protons, indicating that the rat segment 128-136, although necessary, is not sufficient to confer proton-sensitivity.

Thus far, the above chimeras demonstrate that rat sequences in the extracellular domain following TM1 are necessary for the function of the chimeras. Additional chimeras were designed to examine the contribution of the region comprised between residues 346 and 424 in the most distal part of the extracellular domain. CH9 replaced rat residues 346 to 376 with lamprey, which resulted in loss of function. This is a highly conserved area with only a few different residues. Substitutions of these residues with the ones corresponding to rat restored function as indicated by CH11. Three residues were required for full recovery, D_{351}, Q_{358} and E_{359}.

Proton-sensitivity of rat-shark chimeric channels. To test whether the segments of the extracellular domain in rASIC1 identified by rat-lamprey chimeras are important, we made two rat-shark chimeras using the sASIC1α cDNA comparable to rat-lamprey CH2 and CH5. Fig. 7 shows in black and hatched the sequences corresponding to rat and shark in the chimeras. The two constructs were functional, CH1 produced currents similar to rASIC1 but CH2 exhibited kinetics of activation and desensitization much faster than rASIC1.

Sequence analysis of ASICs. Functional studies of ASIC1 from different species have made it apparent that these channels can be separated into proton-sensitive and proton-insensitive types. This raises the question of whether specific residues and/or motifs are responsible for distinguishing between the two groups. Such motifs may form the binding sites for protons or they may mediate conformational changes for channel gating in response to variations in local pH. To identify such residues, we constructed multiple sequence alignment of twelve

known ASIC1 sequences (human-1, rat-1α and -1β, chicken-1, toadfish-1.1 and -1.2, zebrafish-1.1, -1.2 and 1.3, shark-1α and -1β, and lamprey-1) and examined the amino acid compositions at individual positions in the alignment. The aim was to find residues that are either conserved or selected for some amino acids in proton-sensitive but not in proton-insensitive channels, or vice versa. Even though several methods were used (see supplementary data), our analyses did not identify residues with amino acids selected exclusively for the proton-sensitive group. We only found positions significantly enriched with certain types of amino acids in one group but not in the other (red and blue in the sequence alignments in supplementary data). These residues were not only distributed in the extracellular domain, but also in the amino and carboxyterminus.

Subsequently, we examined the sequence conservation of ASIC1 by calculating c-scores, as explained in Methods. The solid and dotted lines in Fig. 8A represent proton-sensitive and -insensitive channels; a low c-score indicates high degree of sequence conservation and high score indicates high variability within the group. Overall the pattern of sequence conservation is similar between the two groups. TM2 and many segments of the ectodomain are identical whereas the first third of the protein and the carboxyterminus are the most variable (c-score 50-90).

Two small regions exhibit different levels of variability between the groups. The region of residues 76-88 in the proton-sensitive type is highly conserved (c-score ~27) whereas in the proton–insensitive type is more variable (c-score ~56). This former region coincides with the segment we identified as important for proton-sensitivity. In contrast the region 289-295 is more conserved in the proton-insensitive (c-score ~20) than in the -sensitive group (c-score

~48). However, functional studies did not implicate this segment as important for proton sensitivity.

To further investigate whether the differences in protein sequence observed in the two groups of ASIC1 reflect changes in natural selection that gave rise to a new functional specialization of the channel, we examined the pace of protein evolution as scaled to neutral divergence approximated by the ratio between nonsynonymous (Ka) and synonymous (Ks) substitution rates in the coding region of the cDNA (Li *et al.* 1997). Two processes have been identified that increase the Ka/Ks ratio (>1); one is positive selection in favor of a change in gene function. The other is relaxed selection.

The average of Ka/Ks ratios in pairwise comparison is 0.13 (0.02~0.19) for proton-sensitive and 0.1 (0.07~0.13) for proton-insensitive, indicating high conservation and functional constraint within each group. Pairwise comparison between proton-sensitive and proton-insensitive ASIC1s gives the average Ka/Ks value 0.14 (0.02~0.55), meaning a high level of conservation between the two groups as well. However, if gain of proton-sensitivity requires changes only in a small domain of the protein, the Ka/Ks analysis using the entire sequence would miss such differences. We thus applied a sliding-window analysis of the Ka/Ks ratio (Fig. 8B). Residues between 184 and 450 show smaller Ka/Ks ratios than residues in other regions thereby they are under strong functional constraint in both groups. A Ka/Ks value >1 in 480-500 could reflect that the carboxyterminus is under positive selection in proton-insensitive channels whereas a value of ~0.4 in proton-sensitive channels is indicative of moderate constraint. The Ka/Ks ratios of residues 64-112 following TM1 are also different between the two groups with a value of ~0.26 for proton-sensitive and 0.53 for proton-insensitive which is indicative of stronger functional constrains in the proton-sensitive group

than in the proton-insensitive group. Together sequence analyses suggest that the highly variable segment after TM1 has become “fixed” in proton-sensitive ASIC1, which in turn may reflect adaptation to a new function.

DISCUSSION

Determinants of proton-sensitivity and gating of ASIC1

Of particular interest is the question regarding what structural elements underlie the differences between proton-sensitive and proton-insensitive ASIC1 channels, and how differences in proton-sensitivity of ASIC1 affect brain functions. Here, we addressed the first question by examining chimeras derived from proton-sensitive and proton-insensitive ASIC1 channels. The studies identified noncontiguous regions in the extracellular domain of rASIC1 that are required to confer proton-sensitivity. The region encompassed by residues 78 to 136 together with a few more distal residues, D_{351}, Q_{358} and E_{359}, from rASIC1 rendered lamprey and shark ASIC1 responsive to protons. However, with the exception of chimeras that contained the whole extracellular domain of rat, all other functional constructs induced channels with kinetics of activation and desensitization different from rASIC1.

A proposed mechanism for ASIC gating is the release of a blocking Ca^{2+} ion from the outer pore (Immke & McCleskey, 2004). Protons compete for this high affinity Ca^{2+}-binding site promoting the release of Ca^{2+} thereby opening the channel. To interpret our results in light of this hypothesis, the first question is whether the regions we identified constitute a Ca^{2+} binding site. Residues D_{351}, Q_{358} and E_{359} could potentially make a high affinity Ca^{2+}-binding site. However, they are not conserved in all proton-sensitive ASIC1s and their substitution by alanine did not abolish proton-sensitivity (data not shown) indicating that, although required, they are not sufficient to confer proton-sensitivity to rat ASIC1. On the other hand, the segment 78-136 is essential although it also does not contain a canonical Ca^{2+}-binding site. Moreover, the variability of amino acid composition of segment 78-136 in both proton-sensitive and proton-insensitive groups makes it unlikely to encode for the Ca^{2+}-binding site.

On the other hand, proton-sensitive chimeras exhibited marked differences in kinetics of activation and desensitization suggesting that the regions identified as functionally important do not form a Ca^{2+}-binding site but rather are involved in conformational changes elicited upon proton binding.

Evolution of proton sensitivity of ASIC1

Gating by external protons is considered to be the most distinct feature of the ASICs. Indeed, it was this property that gave to ASIC1 the name of acid-sensing ion channel. Although in most instances the physiological role of the ASICs has not been completely elucidated, the underlying notion is that protons are required for activation of the ASICs in nociceptors, synaptic terminals of central neurons and in pathological conditions such as brain injury induced by ischemia. The results of this study challenge that notion by demonstrating that proton-sensitivity is not a general property of ASIC1, it was acquired late in the evolution of vertebrates with the rise of bony fishes, some 400 million years ago. This finding has important functional implications for ASIC1, which is the prototype and the most abundant and ubiquitous channel of the ASIC family. Indeed, inactivation of the ASIC1 gene in mice abolishes proton-activated currents in many regions of the mammalian brain indicating that it forms homomeric channels that are not substituted by channels with other subunit compositions (Wemmie *et al.* 2002).

It is remarkable that, in spite of the high degree of amino acid conservation of ASIC1 through the whole chordate lineage (evolutionary time spanning more than 550 million years), there is such a stark difference regarding proton-sensitivity. The findings presented here imply that ASIC1 from lower vertebrates is gated by a stimulus different from protons.

Whether ASIC1s of recent vertebrates still respond to the primordial agonist is not known but raises the possibility that mammalian ASIC1 may be gated *in vivo* by agonists other than protons.

One interpretation of these results is that proton-sensitivity arose in evolution to provide a new function or to improve a previously existing one (such as nociception or long term potentiation in higher vertebrates). This interpretation predicts that the residues conferring proton-sensitivity would become fixed in the protein; however, the group of proton-sensitive ASIC1s could not be distinguished from the proton-insensitive group by any signature in the protein sequence. On the other hand, the relatively high Ka/Ks ratio of the 78-136 segment in the proton-insensitive group (Fig. 8B) likely provided conditions for emergence of amino acids combinations that support proton-sensitivity. The subsequent decrease in Ka/Ks ratio of this segment is consistent with proton-sensitivity conferring some evolutionary advantage. Alternatively, proton sensitivity may not be directly relevant to the functional specialization of the protein because variation in proton-sensitivity might be driven by near neutral evolution, and will be tolerated because it does not hamper the survival of the species. Further characterization of the functional role of ASIC1 in early and more recent vertebrates will provide a definitive answer.

Figure Legends

Figure 1. Phylogenetic analysis of ASIC gene family generated using MEGA version 2.1 (Kumar *et al*. 2001). Tree for the phylogram was established by Neighbor-Joining, with the alignment form ClustalX. The tree was rooted with FaNaC as an outgroup. The bar represents genetic distance in substitutions per amino-acid. New sequences reported in this work are in bold. The corresponding nucleotide sequences have been submitted to GeneBank; the accession numbers are AY278028, AY275841, AY275840, AY956390, AY956391, AY956392 and AY956393 for ASIC 1-toadfish, 2-toadfish, 1.2-toadfish, 1-lamprey, 1a-shark, 1b-shark and 1-chicken, respectively.

Figure 2. Proton-gated currents of fish ASICs. Whole-cell currents elicited by a change in pH_o from 7.4 to 5.0 (bars above the current traces) were recorded with TEVC from injected oocytes. The bath solution contained 150 mM NaCl and the membrane potential was held at -60 mV. A) Representative examples of current trances from oocytes injected with single cRNAs corresponding to each of the toadfish ASICs, and oocytes injected with the indicated combinations of cRNAs. B) Relative currents induced by pH_o 5.0. The data represent the summary of five independent experiments where the peak currents were normalized to the values of fASIC1. Error bars are the standard deviation, n for each column ≥8 oocytes. All conditions were statistically different from fASIC1 ($p< 0.001$).

Figure 3. A) Identification of fASIC1-HA, fASIC2-FLAG and fASIC1.2-FLAG from injected oocytes by western blotting using HA and FLAG monoclonal antibodies. $fASIC2_{G431F}$ and $fASIC1.2_{G458F}$ mutants are also shown. B) Western blotting of surface

biotinylated proteins of cells expressing toadfish ASIC1.2, ASIC2 or rat ASIC1 revealed with anti-FLAG monoclonal. C) Co-immunoprecipitation of fish ASICs from injected *Xenopus* oocytes. Oocytes were injected with equal amounts of cRNAs from fASIC1+fASIC2 or fASIC1+fASIC1.2. Two days after injection oocytes were examined for proton-gated currents and saved for co-immunoprecipitation with anti-HA rabbit polyclonal antibody under non-denaturing conditions. Immunecomplexes were resolved on SDS-PAGE and then transferred to membranes for western blotting. Signals corresponding to fASIC2 and fASIC1.2 were detected with anti-FLAG monoclonal antibody. Arrows on the left margin of the gels indicate molecular weight markers.

Figure 4. Functional expression of homomeric shark or lamprey channels and heteromeric rat/shark and rat/lamprey ASIC1 channels in *Xenopus* oocytes. A) Three days after injection of oocytes with cRNA of rat and/or sASIC1α in ratios indicated below the columns were examined for expression of proton-induced currents with the TEVC. Oocytes were exposed to a preconditioning solution of pH_o 7.4 and activated with pH_o 4.0 for 5 s. The mean of the values±SD of whole-cell currents (μA/oocyte) are shown in the ordinate (n=8). B) Similar experiments conducted with oocytes injected with rat and/or lamprey shark cRNAs.

Figure 5. Expression of shark and lamprey ASIC1 in the plasma membrane of oocytes. Western blot of oocytes injected with shark or lamprey ASIC1 cRNA. Oocytes were first treated with sulfo-NHS-SS-Biotin and the biotinylated proteins were isolated with streptavidine beads. Total cell lysate (Tot) and biotinylated proteins (Mem) were resolved in

10% SDS-PAGE, transferred to membranes and processed for western blotting with V5 (shark) or FLAG (lamprey) monoclonal antibodies, respectively.

Figure 6. Rat-lamprey chimeras. A) Alignment of rat and lamprey ASIC1. Identical residues are shown in red and different ones in black. Numbers above the sequence correspond to the amino acid positions in the lamprey sequence. Boxes enclose TM1 and TM2. B) Schematic representation of rat-lamprey CHs. Sequences corresponding to rat and lamprey are indicated in black and white, respectively. The names of the chimeras are on the left. The numbers over each chimera are the same as in A. On the right is indicated the response to a pulse of pH_o 4.0. C) Representative current traces of oocytes expressing proton-sensitive CHs activated by pH_o 4.0 indicated by the bars over the current traces. The time and current scale bars under each trace correspond to 1 s and 1 μA. D) Western blots of surface biotinylated CHs (Membrane) and a fraction of cell lysate (Total) revealed by anti-FLAG monoclonal antibody.

Figure 7. Rat-shark1α chimeras. A) Schematic of rat-shark1α CHs. Black and hatched represent sequences from rat and shark respectively. Numbers are amino acid positions in the shark sequence. B) Representative examples of whole-cell currents elicited by changes in pH_o from 7.4 to 4.0 (bar above traces) obtained with TEVC. Bars indicate current and time scales.

Figure 8. Sequence analysis of proton-sensitive and -insensitive ASIC1s. A) Conservation of amino acid composition within proton-sensitive (solid line) and -insensitive (dotted line)

ASIC1s assessed by c-scores. A low score indicates that a region is conserved. B) Sliding window analysis of Ka/Ks ratios performed on proton-sensitive (solid line) and proton-insensitive (dotted line) ASIC1s. Window of 90 nt with 12 nt step was used for the study. The x-axis indicates the residue numbered according to the rat ASIC1α sequence. Protein domains are drawn schematically above each graph with transmembrane segments shown in black. Regions that were found important for proton-sensitivity in functional studies are shaded in gray.

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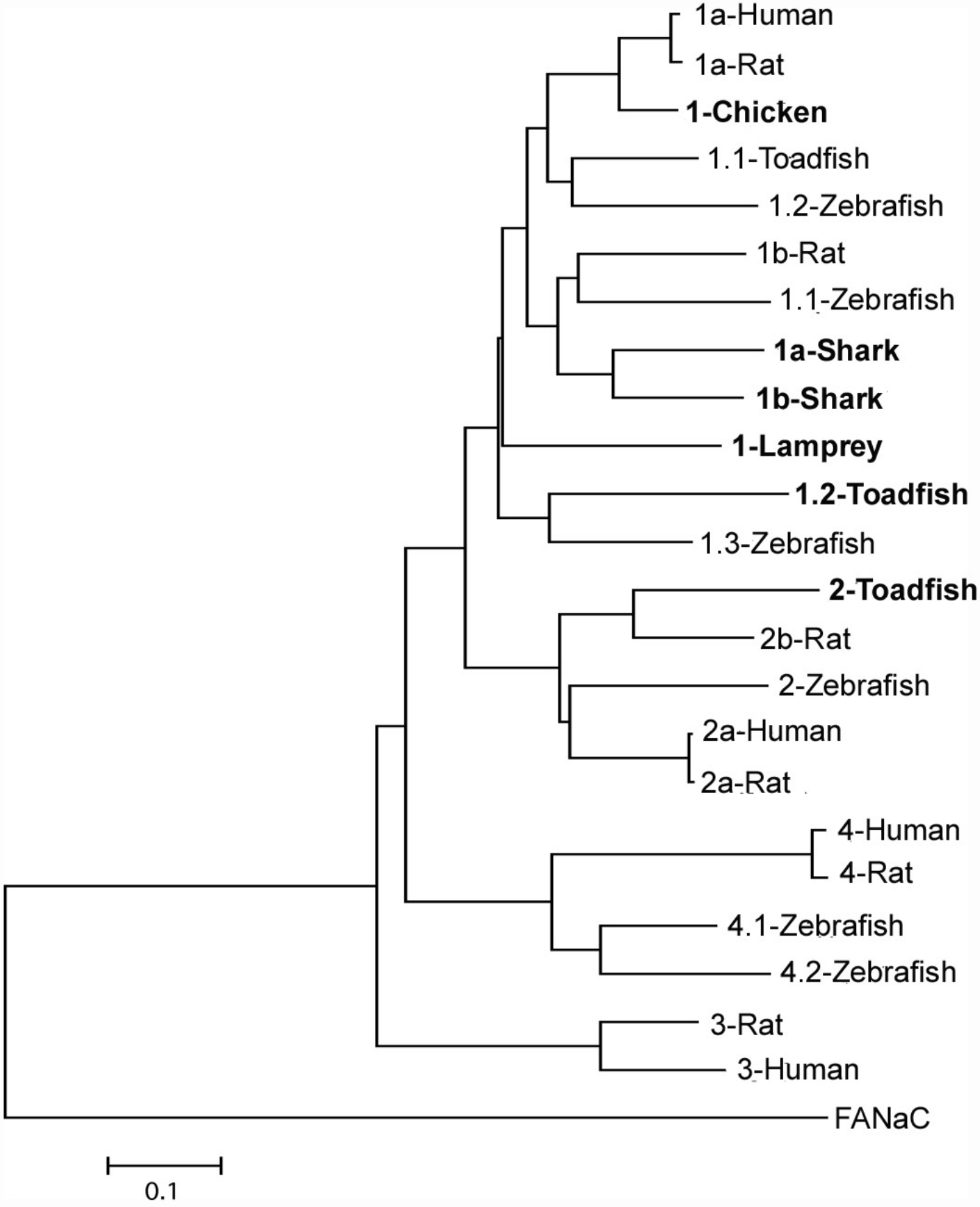

1a-Human
1a-Rat
1-Chicken
1.1-Toadfish
1.2-Zebrafish
1b-Rat
1.1-Zebrafish
1a-Shark
1b-Shark
1-Lamprey
1.2-Toadfish
1.3-Zebrafish
2-Toadfish
2b-Rat
2-Zebrafish
2a-Human
2a-Rat
4-Human
4-Rat
4.1-Zebrafish
4.2-Zebrafish
3-Rat
3-Human
FANaC
0.1

Figure 1

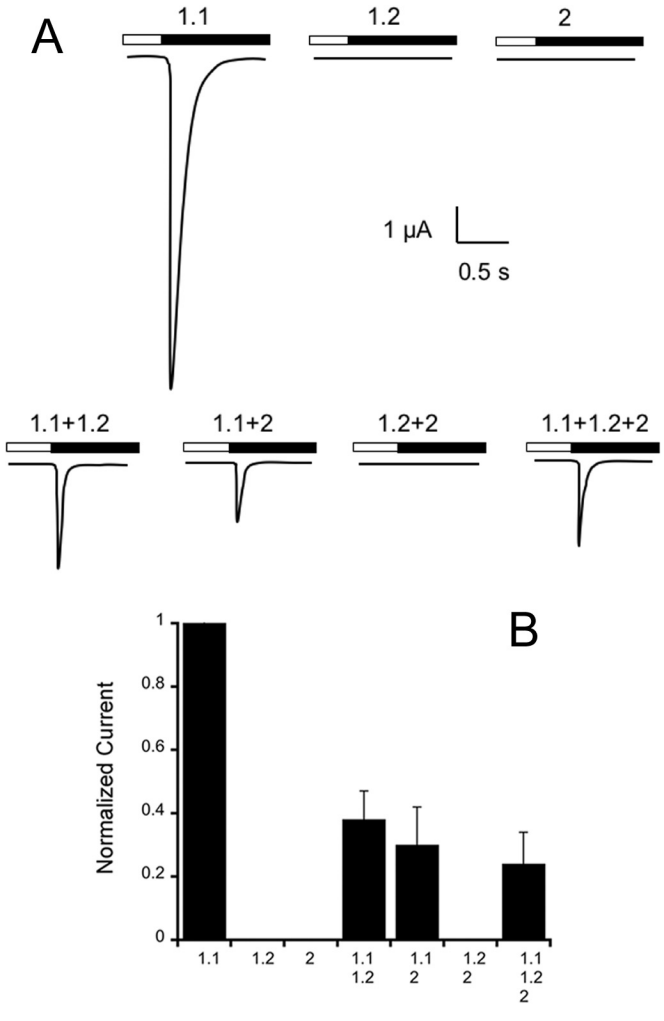

A
1.1
1.2
2
1 μA
0.5 s
1.1+1.2
1.1+2
1.2+2
1.1+1.2+2
B
Normalized Current
1
0.8
0.6
0.4
0.2
0
1.1
1.2
2
1.1 1.2
1.1 2
1.2 2
1.1 1.2 2

Figure 2

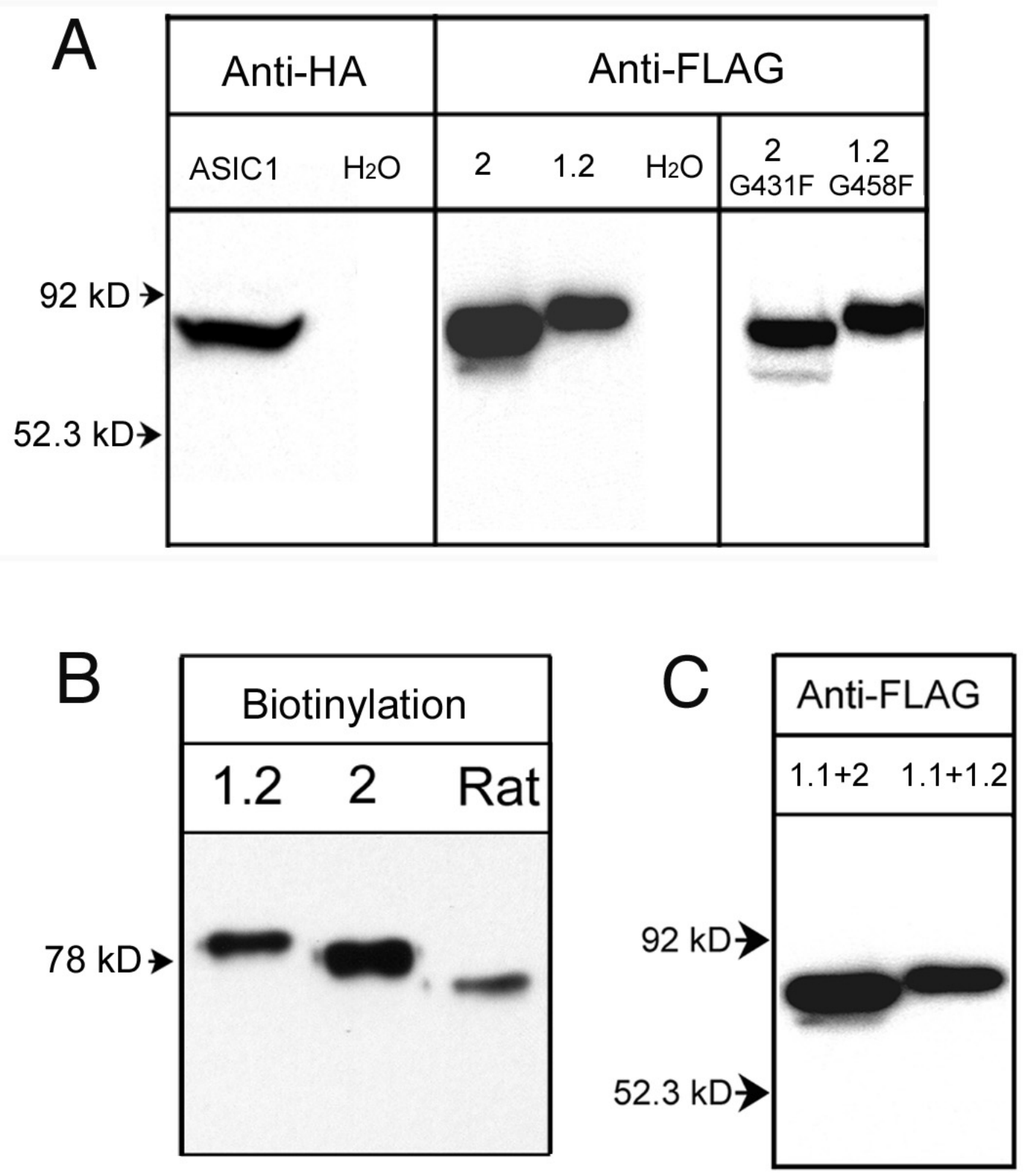

A
Anti-HA
Anti-FLAG
ASIC1
H2O
2
1.2
H2O
2 G431F
1.2 G458F
92 kD
52.3 kD
B
Biotinylation
1.2
2
Rat
78 kD
C
Anti-FLAG
1.1+2
1.1+1.2
92 kD
52.3 kD

Figure 3

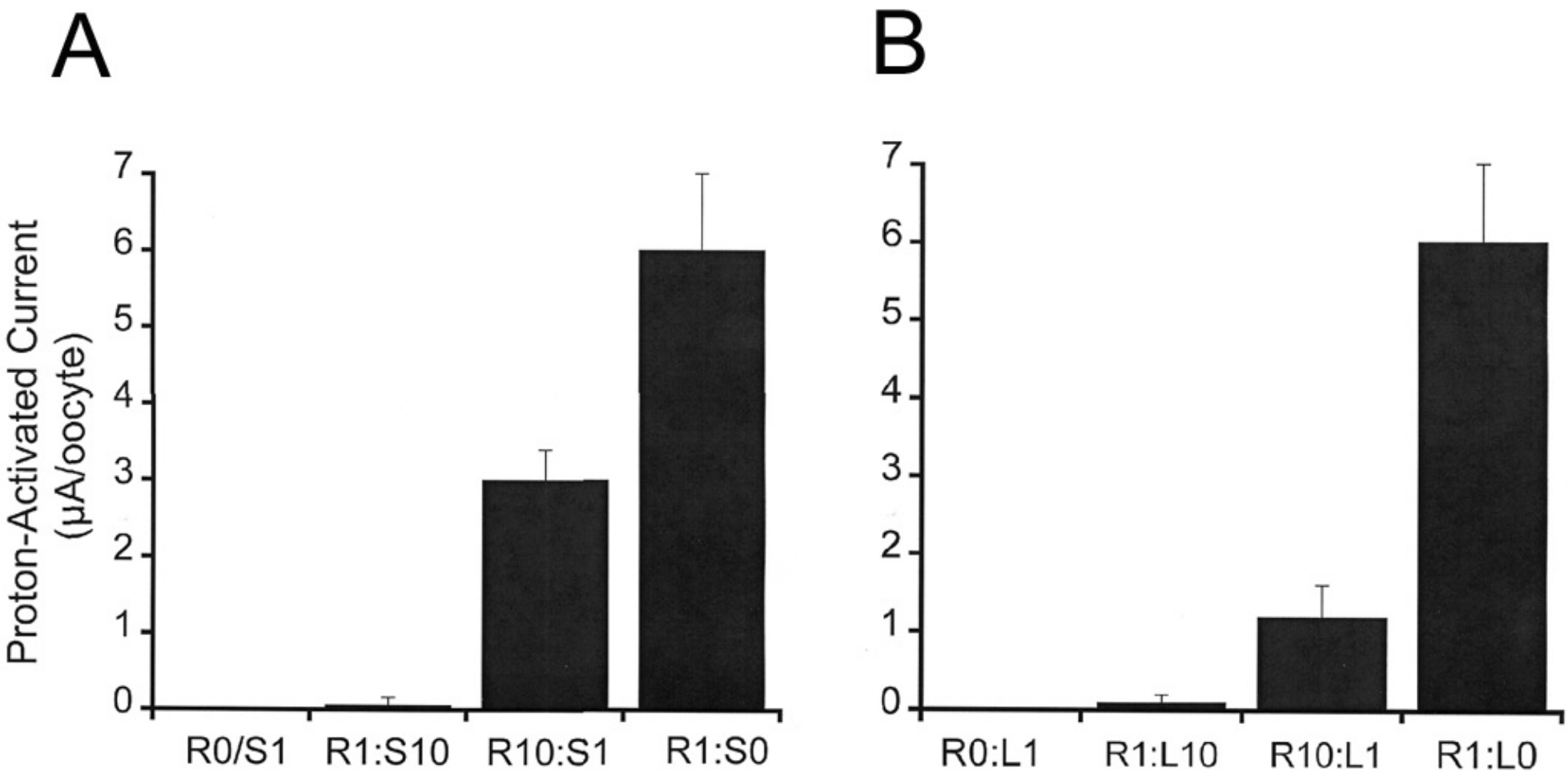

A
B
Proton-Activated Current (μA/oocyte)
0
1
2
3
4
5
6
7
R0/S1
R1:S10
R10:S1
R1:S0
R0:L1
R1:L10
R10:L1
R1:L0

Figure 4

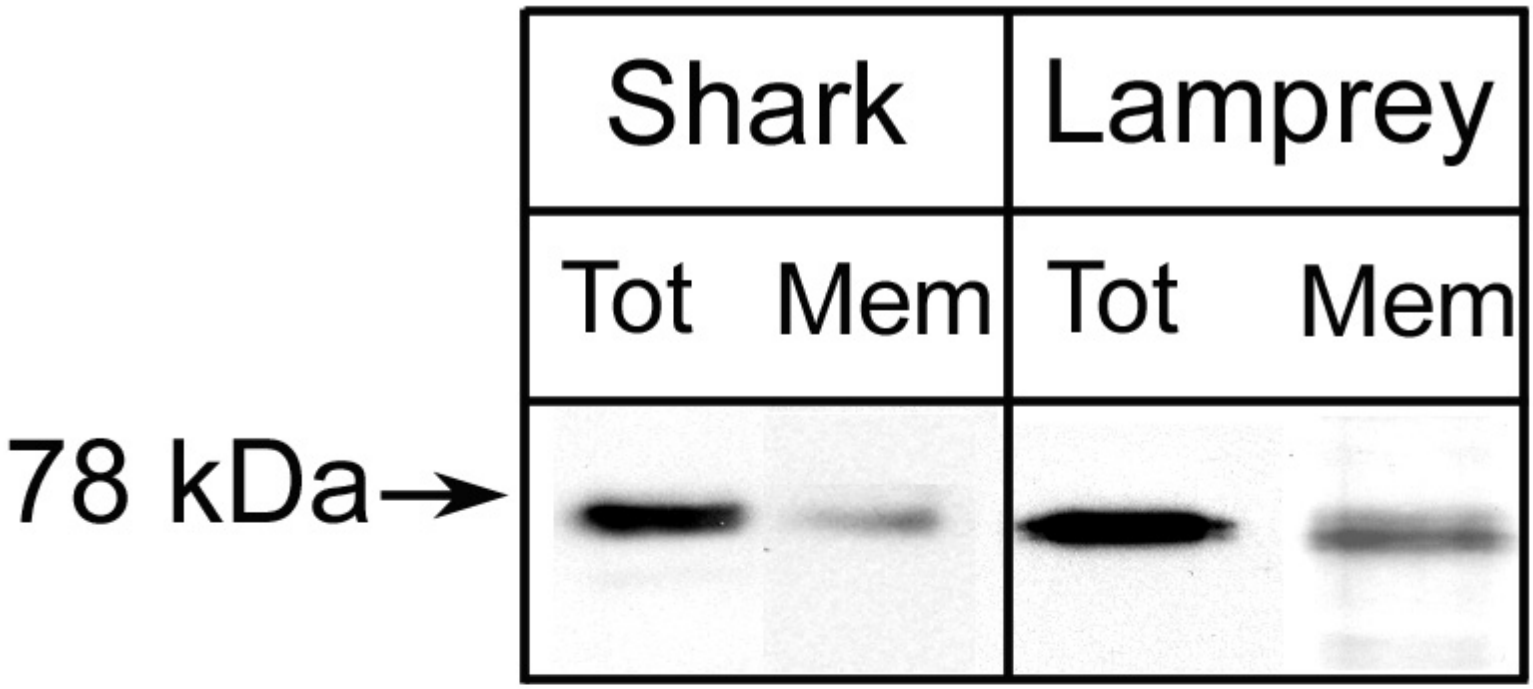


Figure 5

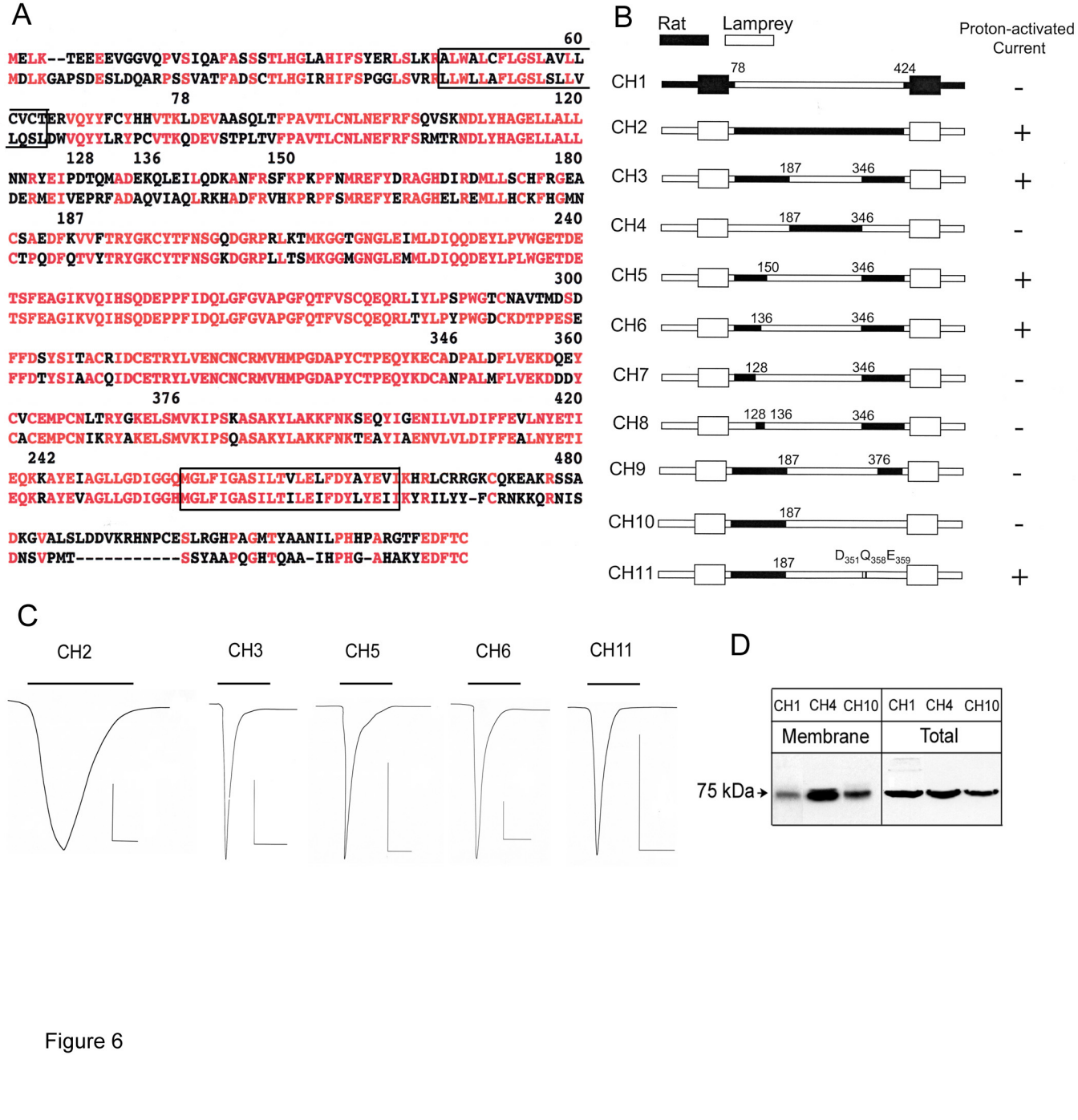

A
B
Rat
Lamprey
Proton-activated Current
CH1
CH2
CH3
CH4
CH5
CH6
CH7
CH8
CH9
CH10
CH11
78
424
187
346
150
136
128
376
$D_{351}Q_{358}E_{359}$
C
CH2
CH3
CH5
CH6
CH11
D
CH1 CH4 CH10 CH1 CH4 CH10
Membrane
Total
75 kDa

Figure 6

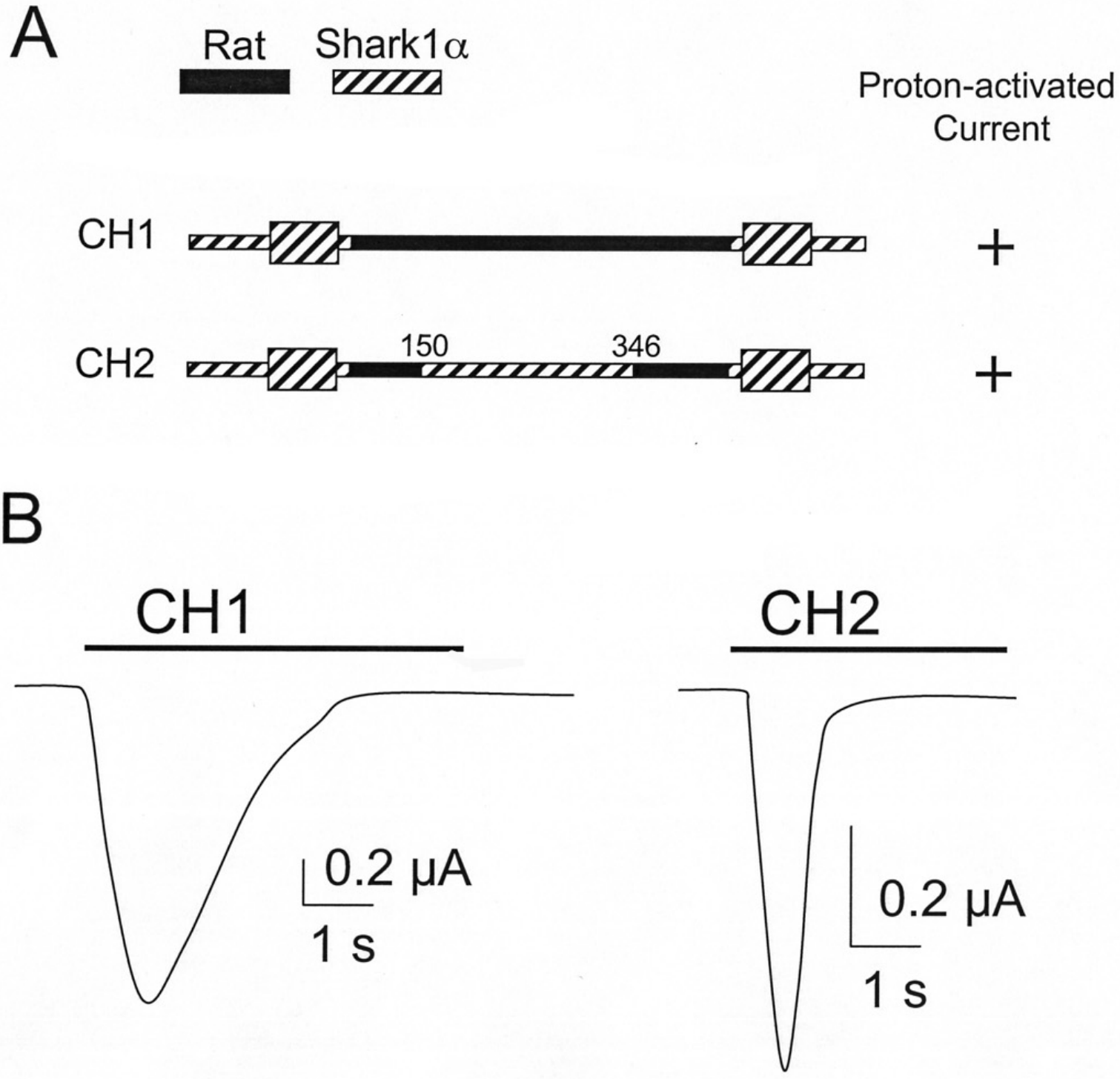

A
Rat
Shark1α
Proton-activated Current
CH1
+
CH2
150
346
+
B
CH1
CH2
0.2 μA
1 s
0.2 μA
1 s

Figure 7

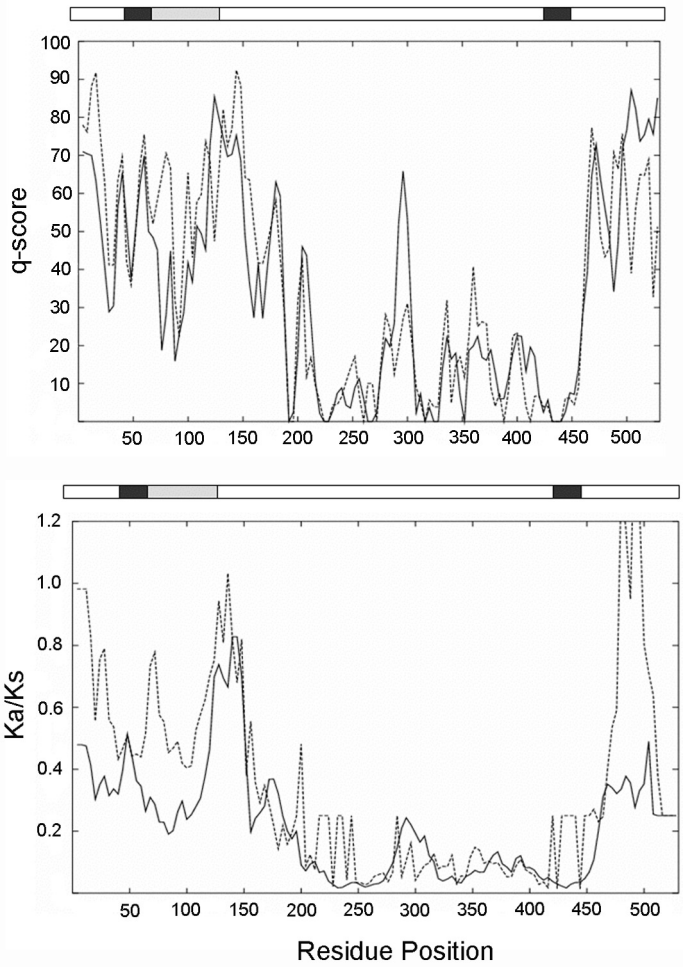
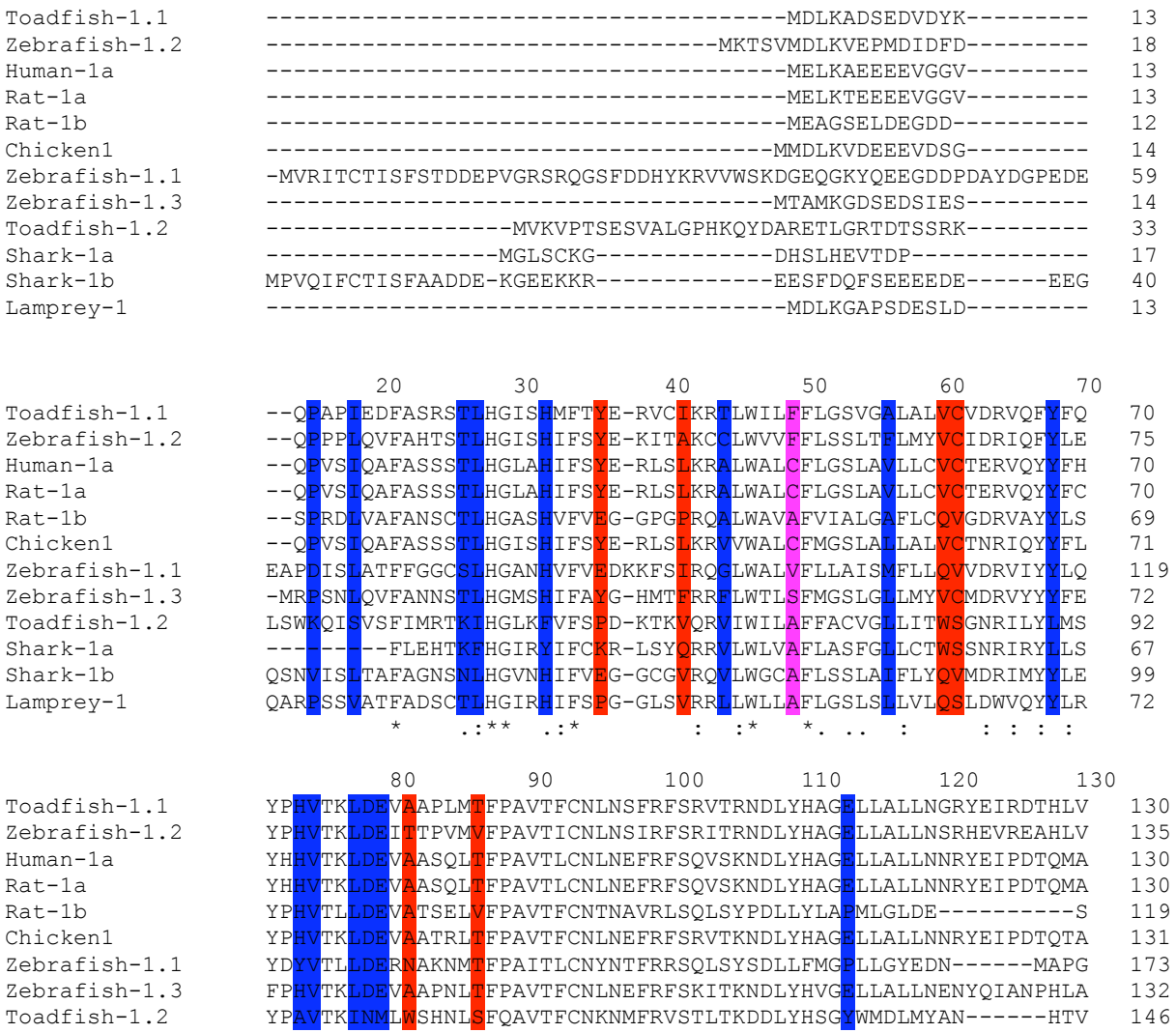

q-score
100
90
80
70
60
50
40
30
20
10
50
100
150
200
250
300
350
400
450
500
Ka/Ks
1.2
1.0
0.8
0.6
0.4
0.2
50
100
150
200
250
300
350
400
450
500
Residue Position

Figure 8

SUPPLEMENT

Two methods were implemented to identify regions in which the two groups of ASIC1 proteins display different sequence features. **Method 1:** Amino acid composition profiles were generated to represent the amino acid frequencies in each column of the multiple sequence alignment (MSA), one for proton-sensitive and one for proton-insensitive ASIC1s. For each column, we computed the distance between the two profiles; a small value indicates that the 20 amino acids were observed with similar frequencies between the two groups. Values derived from individual columns in the MSA were then compared against the average of all positions in the sequence alignment. Residues with values two standard deviations away from the mean are colored in red; they correspond to positions where the two groups of ASIC1s have significantly different preference of amino acids. **Method 2.** For each column of the MSA, we computed a similarity score according to physicochemical properties (e.g., aliphatic, charge, polar) of amino acids, one for proton-sensitive and one for proton-insensitive ASIC1s and then computed the difference of the two scores. A small value indicates that both groups of ASIC1 show similar sequence variation in a column, i.e., either both are conserved or both are diverse. (If they are both conserved, they can have different preferences of amino acids since the score is just a measurement of *similarity within each group*). These values of individual columns in the MSA were then compared against the average derived from all positions in the sequence alignment. Residues with values two standard deviations away from the mean are colored blue. They correspond to positions where one group of ASIC1s is conserved but the other is not. Residues that were found significantly different by both methods are colored purple.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Zebrafish-1.2 ----------MKTSVMDLKVEPMDIDFD--------- 18
Human-1a ----------MELKAEEEEVGGV--------- 13
Rat-1a ----------MELKTEEEEVGGV--------- 13
Rat-1b ----------MEAGSELDEGDD---------- 12
Chicken1 ----------MMDLKVDEEEVDSG--------- 14
Zebrafish-1.1 -MVRITCTISFSTDDEPVGRSRQGSFDDHYKRVVWSKDGEQGKYQEEGDDPDAYDGPEDE 59
Zebrafish-1.3 ----------MTAMKGDSEDSIES--------- 14
Toadfish-1.2 ----------MVKVPTSESVALGPHKQYDARETLGRTDTSSRK--------- 33
Shark-1a ----------MGLSCKG----------DHSLHEVTDP---------- 17
Shark-1b MPVQIFCTISFAADDE-KGEEKKR----------EESFDQFSEEEEDE------EEG 40
Lamprey-1 ----------MDLKGAPSDESLD--------- 13
20 30 40 50 60 70
Toadfish-1.1 --QPAPIEDFASRSTLHGISHMFTYE-RVCIKRWLWILFFLGSVGALALVCVDRVQFYFQ 70
Zebrafish-1.2 --QPPPLQVFAHTSTLHGISHIFSYE-KITAKCCLWVVFFLSSLTFLMYVCIDRIQFYLE 75
Human-1a --QPVSIQAFASSSTLHGLAHIFSYE-RLSLKRALWALCFLGSLAVLLCVCTERVQYYFH 70
Rat-1a --QPVSIQAFASSSTLHGLAHIFSYE-RLSLKRALWALCFLGSLAVLLCVCTERVQYYFC 70
Rat-1b --SPRDLVAFANSCTLHGASHVFVEG-GPGPRQALWAVAFVIALGAFLCQVGDRVAYYLS 69
Chicken1 --QPVSIQAFASSSTLHGISHIFSYE-RLSLKRVVWALCFMGSLALLALVCTNRIQYYFL 71
Zebrafish-1.1 EAPDISLATFFGGCSLHGANHVFVEDKKFSIRQCLWALVFLLAISMFLLQVVDRVIYYLQ 119
Zebrafish-1.3 -MRPSNLQVFANNSTLHGMSHIFAYG-HMTFRRFLWTLSFMGSLGLLMYVCMDRVYYYFE 72
Toadfish-1.2 LSWKQISVSFIMRTKIHGLKFVFSFD-KTKVQRVIWILAFFACVGLLITWSGNRILYLMS 92
Shark-1a ---------FLEHTKFHGIRYIFCKR-LSYQRRVLWLVAFLASFGLLCTWSSNRIRYLLS 67
Shark-1b QSNVISLTAFAGNSNLHGVNHIFVEG-GCGVRQVLWGCAFLSSLAIFLYQVMDRIMYYLE 99
Lamprey-1 QARPSSVATFADSCTLHGIRHIFSFG-GLSVRRLLWLLAFLGSLSLLVLQSLDWVQYYLR 72
* .:** .:* : :* *. .. : : : :
80 90 100 110 120 130
Toadfish-1.1 YPHVTKLDEVAAPLMTFPAVTFCNLNSFRFSRVTRNDLYHAGLLLALLNGRYEIRDTHLV 130
Zebrafish-1.2 YPHVTKLDEITTPVMVFPAVTICNLNSIRFSRITRNDLYHAGLLLALLNSRHEVREAHLV 135
Human-1a YHHVTKLDEVAASQLTFPAVTLCNLNEFRFSQVSKNDLYHAGLLLALLNNRYEIPDTQMA 130
Rat-1a YHHVTKLDEVAASQLTFPAVTLCNLNEFRFSQVSKNDLYHAGLLLALLNNRYEIPDTQMA 130
Rat-1b YPHVTLLDEVATSELVFPAVTFCNTNAVRLSQLSYPDLLYLAPMLGLDE----------S 119
Chicken1 YPHVTKLDEVAATRLTFPAVTFCNLNEFRFSRVTKNDLYHAGLLLALLNNRYEIPDTQTA 131
Zebrafish-1.1 YDYVTLLDERNAKNMTFPAITLCNYNTFRRSQLSYSDLLFMGPLLGYEDN------MAPG 173
Zebrafish-1.3 FPHVTKLDEVAAPNLTFPAVTFCNLNEFRFSKITKNDLYHVGLLLALLNENYQIANPHLA 132
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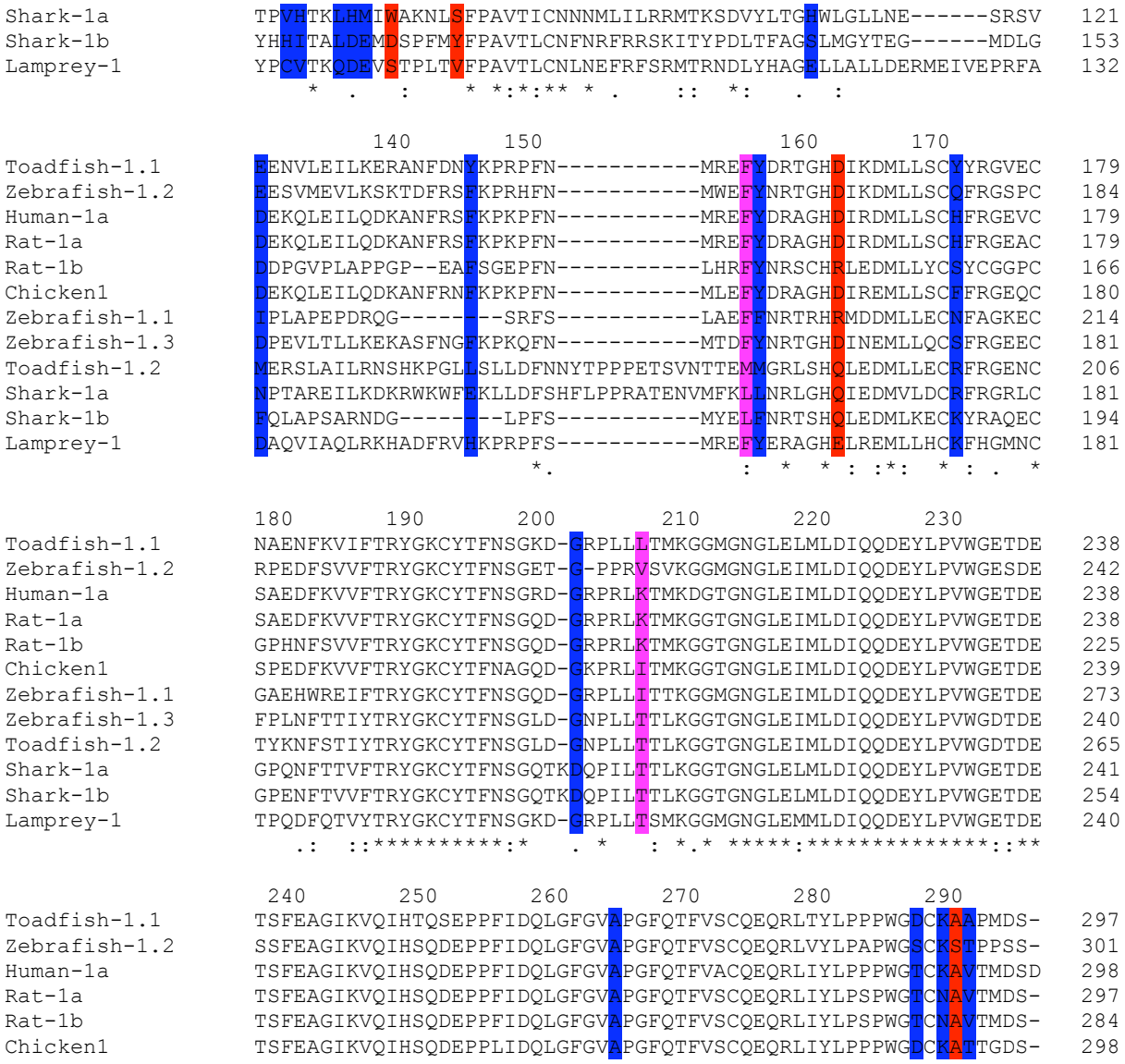
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Shark-1b YHHITALDEMDSPFMYFPAVTLCNFNRFRRSKITYPDLTFAGSLMGYTEG------MDLG 153
Lamprey-1 YPCVTKQDEVSTPLTVFPAVTLCNLNEFRFSRMTRNDLYHAGELLALLDERMEIVEPRFA 132
140 150 160 170
Toadfish-1.1 EENVLEILKERANFDNYKPRPFN----------MREFYDRTGHDIKDMLLSCYYRGVEC 179
Zebrafish-1.2 EESVMEVLKSKTDFRSFKPRHFN----------MWEFYNRTGHDIKDMLLSCQFRGSPC 184
Human-1a DEKQLEILQDKANFRSFKPKPFN----------MREFYDRAGHDIRDMLLSCHFRGEVC 179
Rat-1a DEKQLEILQDKANFRSFKPKPFN----------MREFYDRAGHDIRDMLLSCHFRGEAC 179
Rat-1b DDPGVPLAPPGP--EAFSGEPFN----------LHRFYNRSCHRLEDMLLYCSYCGGPC 166
Chicken1 DEKQLEILQDKANFRNFKPKPFN----------MLEFYDRAGHDIREMLLSCFFRGEQC 180
Zebrafish-1.1 IPLAPEPDRQG--------SRFS----------LAEFFNRTRHRMDDMLLECNFAGKEC 214
Zebrafish-1.3 DPEVLTLLKEKASFNGFKPKQFN----------MTDFYNRTGHDINEMLLQCSFRGEEC 181
Toadfish-1.2 MERSLAILRNSHKPGLLSLLDFNNYTPPPETSVNTTEMMGRLSHQLEDMLLECRFRGENC 206
Shark-1a NPTAREILKDKRWKWFFKLLDFSHFLPPRATENVMFKLLNRLGHQIEDMVLDCRFRGRLC 181
Shark-1b FQLAPSARNDG--------LPFS----------MYELFNRTSHQLEDMLKECKYRAQEC 194
Lamprey-1 DAQVIAQLRKHADFRVHKPRPFS----------MREFYERAGHELREMLLHCKFHGMNC 181
180 190 200 210 220 230
Toadfish-1.1 NAENFKVIFTRYGKCYTFNSGKD-GRPLLLTMKGGMGNGLELMLDIQQDEYLPVWGETDE 238
Zebrafish-1.2 RPEDFSVVFTRYGKCYTFNSGET-G-PPRVSVKGGMGNGLEIMLDIQQDEYLPVWGESDE 242
Human-1a SAEDFKVVFTRYGKCYTFNSGRD-GRPRLKTMKDGTGNGLEIMLDIQQDEYLPVWGETDE 238
Rat-1a SAEDFKVVFTRYGKCYTFNSGQD-GRPRLKTMKGGTGNGLEIMLDIQQDEYLPVWGETDE 238
Rat-1b GPHNFSVVFTRYGKCYTFNSGQD-GRPRLKTMKGGTGNGLEIMLDIQQDEYLPVWGETDE 225
Chicken1 SPEDFKVVFTRYGKCYTFNAGQD-GKPRLITMKGGTGNGLEIMLDIQQDEYLPVWGETDE 239
Zebrafish-1.1 GAEHWREIFTRYGKCYTFNSGQD-GRPLLITTKGGMGNGLEIMLDIQQDEYLPVWGETDE 273
Zebrafish-1.3 FPLNFTTIYTRYGKCYTFNSGLD-GNPLLTTLKGGTGNGLEIMLDIQQDEYLPVWGDTDE 240
Toadfish-1.2 TYKNFSTIYTRYGKCYTFNSGLD-GNPLLTTLKGGTGNGLEIMLDIQQDEYLPVWGDTDE 265
Shark-1a GPQNFTTVFTRYGKCYTFNSGQTKDQPILTTLKGGTGNGLELMLDIQQDEYLPVWGETDE 241
Shark-1b GPENFTVVFTRYGKCYTFNSGQTKDQPILTTLKGGTGNGLELMLDIQQDEYLPVWGETDE 254
Lamprey-1 TPQDFQTVYTRYGKCYTFNSGKD-GRPLLTSMKGGMGNGLEMMLDIQQDEYLPVWGETDE 240
240 250 260 270 280 290
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Zebrafish-1.2 SSFEAGIKVQIHSQDEPPFIDQLGFGVAPGFQTFVSCQEQRLVYLPAPWGSCKSTPPSS- 301
Human-1a TSFEAGIKVQIHSQDEPPFIDQLGFGVAPGFQTFVACQEQRLIYLPPPWGTCKAVTMDSD 298
Rat-1a TSFEAGIKVQIHSQDEPPFIDQLGFGVAPGFQTFVSCQEQRLIYLPSPWGTCNAVTMDS- 297
Rat-1b TSFEAGIKVQIHSQDEPPFIDQLGFGVAPGFQTFVSCQEQRLIYLPSPWGTCNAVTMDS- 284
Chicken1 TSFEAGIKVQIHSQDEPPLIDQLGFGVAPGFQTFVSCQEQRLIYLPPPWGDCKATTGDS- 298

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Zebrafish-1.1      TTFEAGIKVQIHTQDEPPFIDQLGFGVAPGFQTFVSCQEQRLTYLPPPWGDCKATPIDS-   332
Zebrafish-1.3      TSYEAGIKVQIHSQDEPPFIDQLGFGVAPGFQTFVSCQQQLLLYLPPPWGDCRSAPMDS-   299
Toadfish-1.2       TSYEAGIKVQIHSQEEPPFIDQLGFGVAPGFQTFVSCQQQLLQYLPPPWGDCKSTPIDS-   324
Shark-1a           TSFEAGIKVQIHGQNEPPFIDQLGFGVTPGFQTFVSCQEQRLIYLPPPWGDCKSTPMDS-   300
Shark-1b           TSFEAGIKVQIHGQNEPPFIDQLGFGVTPGFQTFVSCQEQRLIYLPPPWGDCKSTPMDS-   313
Lamprey-1          TSFEAGIRVQIHSQDEPPFIDQLGFGVAPGFQTFVSCQEQRLTYLPYPWGDCKDTPPES-   299
                   :::****:**** *.***:********.*******:**:* * *** *** *. .. .*

                    300       310       320       330       340       350
Toadfish-1.1       -DFFSTYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVERDN   356
Zebrafish-1.2      -DYFRAYSISACRTDCETRYLVENCNCRMVHMPGDAPYCTPVLYKECAHPALDFLVETDS   360
Human-1a           LDFFDSYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDQ   358
Rat-1a             -DFFDSYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDQ   356
Rat-1b             -DFFDSYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDQ   343
Chicken1           -EFYDTYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVEKDN   357
Zebrafish-1.1      -DFFNTYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYKECADPALDFLVERDN   391
Zebrafish-1.3      -EYFSTYSITACRIDCETRYLLENCNCRMVHMPGTSTVCTPEQYKDCADPALDFLVEKDN   358
Toadfish-1.2       -EYFSKYSITACRIDCETRYLLENCNCKMVHMPGTSTVCTPEQYKDCADPALDFLVEKDD   383
Shark-1a           -DFFDTYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYRECADPALEFLVEKDS   359
Shark-1b           -DFFDTYSITACRIDCETRYLVENCNCRMVHMPGDAPYCTPEQYRECADPALEFLVEKDS   372
Lamprey-1          -EFFDTYSIAACQIDCETRYLVENCNCRMVHMPGDAPYCTPEQYKECANPALMFLVEKDD   358
                    :::  ***:**: *******:*****:****** :. ***  *::**.*** **** *.

                    360       370       380       390       400       410
Toadfish-1.1       DYCVCKTPCNLTRFGKEMSFVKIPSKASAKYLAKKFNKTEQYIADNILVLDIYFDALNYE   416
Zebrafish-1.2      DYCSCETPCNITRYSKELSFVKIPSKASVKYLAKKYSKSEKYITENVMVLDVFFEALNYE   420
Human-1a           EYCVCEMPCNLTRYGKELSMVKIPSKASAKYLAKKFNKSEQYIGENILVLDIFFEVLNYE   418
Rat-1a             EYCVCEMPCNLTRYGKELSMVKIPSKASAKYLAKKFNKSEQYIGENILVLDIFFEVLNYE   416
Rat-1b             EYCVCEMPCNLTRYGKELSMVKIPSKASAKYLAKKFNKSEQYIGENILVLDIFFEVLNYE   403
Chicken1           EYCVCEMPCNVTRYGKELSMVKIPSKASAKYLAKKYNKSEQYIGENILVLDIFFEALNYE   417
Zebrafish-1.1      DYCVCETPCNMTRYGKELSFVRIPSKASAKYLAKKYNKTEQYISDNIMVLDIFFEALNYE   451
Zebrafish-1.3      DYCVCDTPCNMTRYGKELSMVKIPSKASAKYLAKKFNKTEQYITDNILVLDIFFEALNYE   418
Toadfish-1.2       DYCLCQTPCNTTRYGKELSMVKIPSKASAKYLAKKFNKTEQYIGENILVLDIFFEALNYE   443
Shark-1a           VFCTCETPCNMTRYGKELSMVKIPSKASAKYLAKKYNKSEQYIGENILVLDIFFEALNYE   419
Shark-1b           VFCTCETPCNMTRYGKELSMVKIPSKASAKYLAKKYNKSEQYIGENILVLDIFFEALNYE   432
Lamprey-1          DYCACEMPCNIKRYAKELSMVKVPSQASAKYLAKKFNKTEAYIAENVLVLDIFFEALNYE   418
                    :* *. *** .*:.**:*:*::**:**.******:.*:* ** :*::***::*:.****

                    420       430       440       450       460       470
Toadfish-1.1       TIEQKKAYEVAGLLGDIGGQMGLFIGASILTILELFDYLYEVLKYKLCRCVKKKHKGRGN   476
Zebrafish-1.2      TIEQRKAYEVAGLLGDIGGQMGLFIGASILTILELFDYLYEVMKYRLCRCSNKKHHNNNN   480
Human-1a           TIEQKKAYEIAGLLGDIGGQMGLFIGASILTVLELFDYAYEVIKHKLCRRGKCQKEAKRS   478
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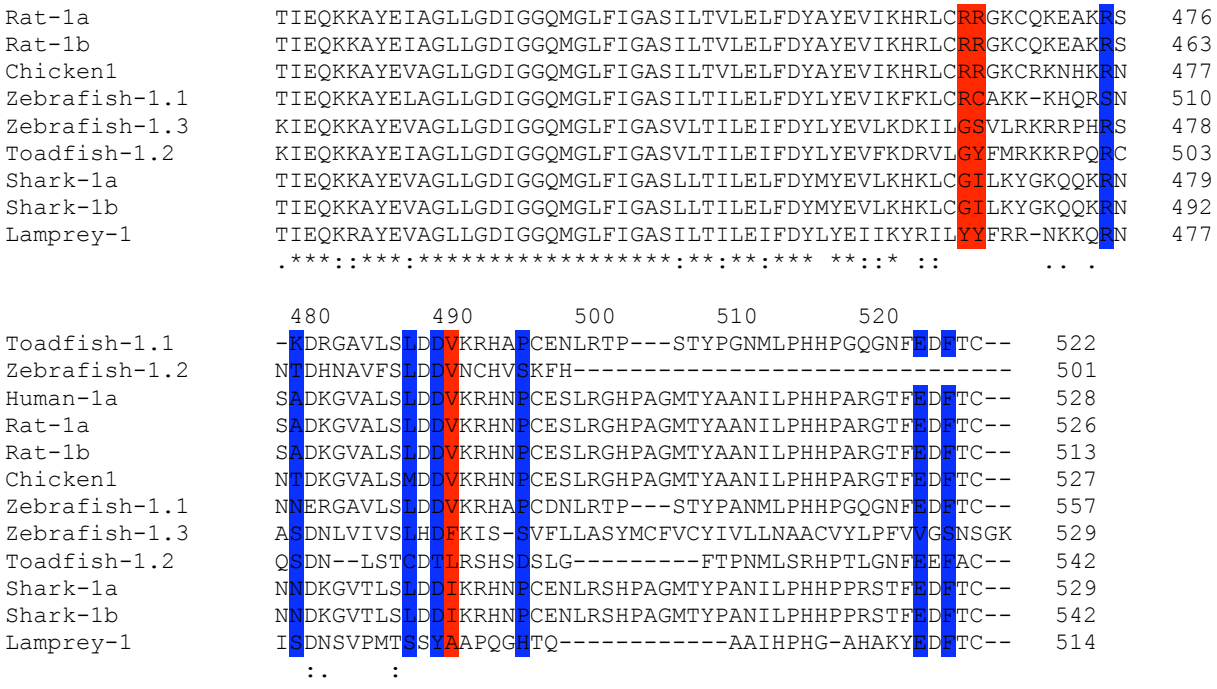
Rat-1a TIEQKKAYEIAGLLGDIGGQMGLFIGASILTVLELFDYAYEVIKHRLCRRGKCQKEAKRS 476
Rat-1b TIEQKKAYEIAGLLGDIGGQMGLFIGASILTVLELFDYAYEVIKHRLCRRGKCQKEAKRS 463
Chicken1 TIEQKKAYEVAGLLGDIGGQMGLFIGASILTVLELFDYAYEVIKHRLCRRGKCRKNHKRN 477
Zebrafish-1.1 TIEQKKAYELAGLLGDIGGQMGLFIGASILTILELFDYLYEVIKFKLCRCAKK-KHQRSN 510
Zebrafish-1.3 KIEQKKAYEVAGLLGDIGGQMGLFIGASVLTILEIFDYLYEVLKDKILGSVLRKRRPHRS 478
Toadfish-1.2 KIEQKKAYEIAGLLGDIGGQMGLFIGASVLTILEIFDYLYEVFKDRVLGYFMRKKRPQRC 503
Shark-1a TIEQKKAYEVAGLLGDIGGQMGLFIGASLLTILELFDYMYEVLKHKLCGILKYGKQQKRN 479
Shark-1b TIEQKKAYEVAGLLGDIGGQMGLFIGASLLTILELFDYMYEVLKHKLCGILKYGKQQKRN 492
Lamprey-1 TIEQKRAYEVAGLLGDIGGQMGLFIGASILTILEIFDYLYEIIKYRILYYFRR-NKKQRN 477
480 490 500 510 520
Toadfish-1.1 -KDRGAVLSLDDVKRHAPCENLRTP---STYPGNMLPHHPGQGNFEDFTC-- 522
Zebrafish-1.2 NTDHNAVFSLDDVNCHVSKFH---------- 501
Human-1a SADKGVALSLDDVKRHNPCESLRGHPAGMTYAANILPHHPARGTFEDFTC-- 528
Rat-1a SADKGVALSLDDVKRHNPCESLRGHPAGMTYAANILPHHPARGTFEDFTC-- 526
Rat-1b SADKGVALSLDDVKRHNPCESLRGHPAGMTYAANILPHHPARGTFEDFTC-- 513
Chicken1 NTDKGVALSMDDVKRHNPCESLRGHPAGMTYAANILPHHPARGTFEDFTC-- 527
Zebrafish-1.1 NNERGAVLSLDDVKRHAPCDNLRTP---STYPANMLPHHPGQGNFEDFTC-- 557
Zebrafish-1.3 ASDNLVIVSLHDFKIS-SVFLLASYMCFVCYIVLLNAACVYLPFVVGSNSGK 529
Toadfish-1.2 QSDN--LSTCDTLRSHSDSLG---------FTPNMLSRHPTLGNFEEFAC-- 542
Shark-1a NNDKGVTLSLDDIKRHNPCENLRSHPAGMTYPANILPHHPPRSTFEDFTC-- 529
Shark-1b NNDKGVTLSLDDIKRHNPCENLRSHPAGMTYPANILPHHPPRSTFEDFTC-- 542
Lamprey-1 ISDNSVPMTSSYAAPQGHTQ----------AAIHPHG-AHAKYEDFTC-- 514