Sequencing the cardiac Na^+-Ca^{2+} exchanger of the spiny dogfish shark (*Squalus acanthias*)

Regina M. Day[1], Hiroko Nagase[1], Young H. Lee[1], Alexander Kraev[2], Lars Cleemann[1], and Martin Morad[1]
[1]Georgetown University, 3900 Reservoir Road, NW, Washington, District of Columbia 20057, USA
[2]Samuel Lunenfeld Research Institute, University of Toronto, 600 University Avenue, Toronto, Canada M5G 1X5

Using samples of mRNA extracted at MDIBL from the heart of the dogfish shark, we sequenced 2688 bp coding 896 aa corresponding to ~87 % of the Na^+-Ca^{2+} exchanger (NCX) as found in other species. The sequenced region corresponds to the regulatory cytoplasmic loop that constitutes the middle portion of the peptide and most of the highly conserved trans-membrane regions that, even without the regulatory loop, support coupled ion translocation. Mammalian NCXs contain tissue-specific structural differences, encoded by a confined variable splicing region, known as A, B, C, D, E, X, and F (Fig. 1A). In contrast, we found that the shark sequence had conspicuous alanine/proline-rich inserts (10 aa and 54 aa) at two other locations (labeled * and **, Fig. 1A). We propose that these inserts may serve as flexible linkers and may be important for the bimodal cAMP-mediated regulation of NCX current that we have observed in shark ventricular cardiomyocytes[7].

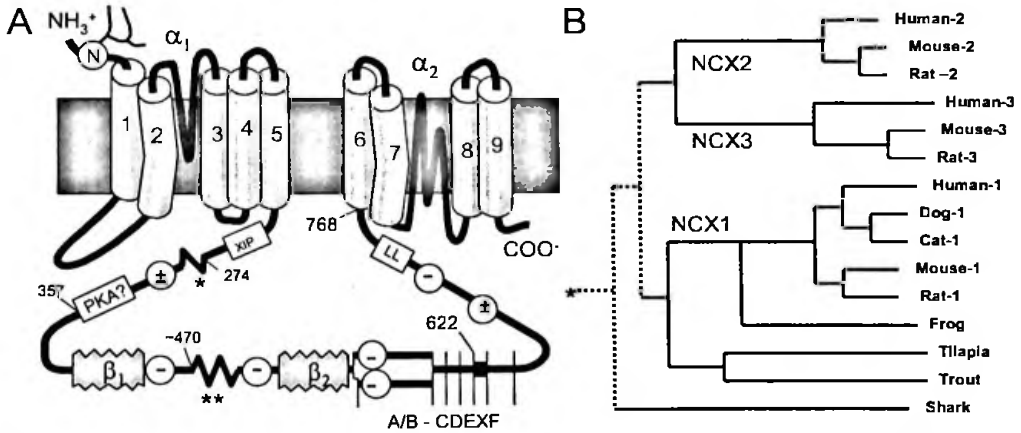

Figure 1. A: Model of NCX composed of 9 trans-membrane segments (1-9) and a long cytoplasmic regulatory loop. The zig-zag lines (labeled * at aa 274 and ** at aa ~470) indicate sites where the incomplete shark sequence has proline/alanine-rich insertions. Numbering of amino acids correspond to dog cardiac NCX1. Modified from [4]. B: Cluster analysis (clustal) of vertebrate NCXs based on conserved bases coding regions near the beginning (377 bp) and end (439 bp) of the mRNA. The phylogenic tree was rooted (*) by squid NCX (U93214-*Loligo*).

In many cells NCX serves mainly to maintain long term Ca^{2+}-homeostasis by steadily extruding Ca^{2+} from the cytosol. In heart cells NCX may additionally provide significant Ca^{2+}-influx during the action potential as well as rapid Ca^{2+} efflux contributing to relaxation during diastole. We have proposed that NCX plays a prominent role in controlling the heartbeat in cardiac cell cells that lack a function sarcoplasmic reticulum (SR), *e.g.* cardiomyocytes from some lower vertebrates (frog, shark) and embryonic and neonate mammals. We have explored how such cells may generate an effective β-adrenergic response in the absence of those targets of cAMP-mediated phosporylation (ryanodine receptors, SR Ca^{2+} ATPase) that are confined to the SR. NCX current (influx and efflux) in frog ventricular cardiomyocytes was suppressed by cAMP, in a manner not seen in adult mammalian cadiomyocytes [2]. Using cloned native and chimeric dog and frog cardiac NCXs we demonstrated that these differences depend on an extra 27 bp exon that in frog NCX encodes a nucleotide binding P-loop (GxxxxGKS, Walker A, exon X in Fig. 1A.) motif within the variable splicing region [3, 5].

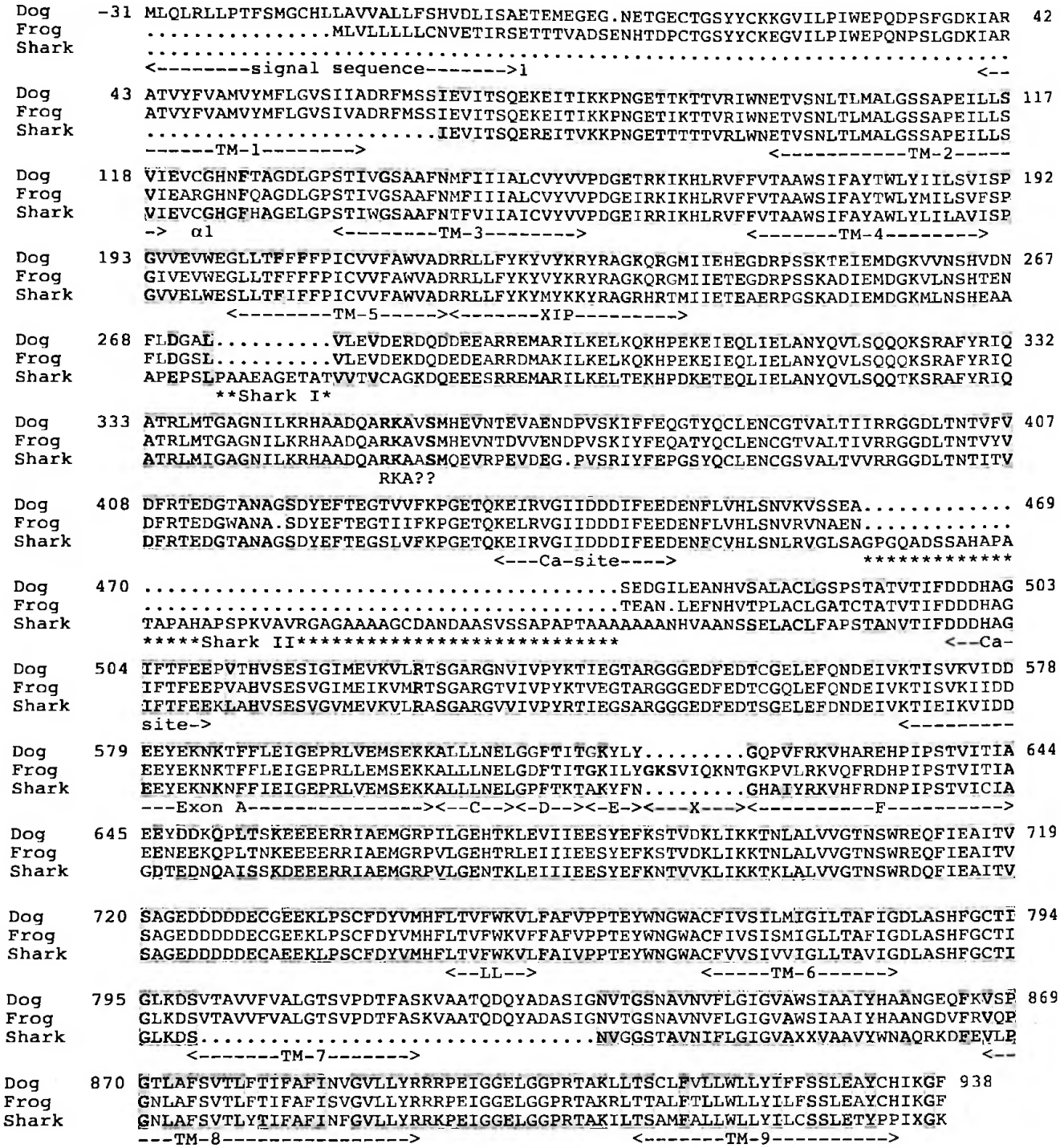

Figure 2. Alignment of the central portion of the NCXs expressed in the hearts of dog, frog and shark. The aa' are numbered according the dog sequence [103], which, except for initial signaling sequence aligns perfectly with the human and feline cardiac NCX1. The one insert for the frog sequence and the two inserts for the shark fall outside this numbering. Shaded aa's are identical or similar (D ≅ E, S ≅ T, K ≅ R, I ≅ L ≅ V). Multalin alignment.

In shark cardiomyocytes a form of cAMP-induced suppression was also present, but the regulation more specifically suppressed the Ca^{2+} influx-mode. Such regulation has been labeled "bimodal". It may serve to prevent Ca^{2+}-overload by limiting Ca^{2+} influx during systole without compromising effective Ca^{2+} extrusion during diastole. To clarify the role and mechanism of bimodal regulation we

have undertaken to sequence and express shark cardiac NCX. The present publication details our progress since we initially sequenced 171 bp [6].

Total RNA was obtained from the heart of the dogfish shark using TRIZOL (Gibco-BRL) and was subjected to reverse transcription using oligo(dT) primers (Invitrogen). For the initial 171 bp amplicon, polymerase chain reaction (PCR) was carried out with primers based on known DNA sequences for the Na^+-Ca^{2+} exchanger from squid and vertebrates (trout, frog and mammals). Bands corresponding to predicted sizes were extracted from TAE agarose gels, and sequenced using the Marine Sequencing Center of the MDIBL; these were then were subcloned into pCR II vector (Invitrogen). A long cDNA fragment was then obtained by PCR from shark RNA using a degenerate 5' primer predicted from the Clustal alignment of 5 vertebrate cDNA sequences of NCX1 and a 3' primer complementary to a portion of the sequenced 171 bp amplicon. The forward primer was 5'-ACCYTKATGGCC-CTGGGWTC-3', where Y=C/T, K=G/T, and W=A/T. The reverse primer was 5'-TGGCAAATGTGTCTGGAACAGA-3'. This resulted in a band at ~2400 bp. The PCR product was sequenced bidirectionally. This larger amplicon spanned 720 amino acids. A final 5' terminal segment of the gene was then cloned using primers designed using the CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers algorhithm (//bioinformatics.weizmann.ac.il/blocks/codehop.html). These primers were forward: 5'-GGCGTGTCCATCATC-GCNGAYMGNTT-3'; reverse: 5'-CCGGGTGGCCTGGAMNCKRTARAA-3', where R=A/G, M=A/C, K=G/T, Y=C/T, and N=A/C/G/T. This PCR resulted in a single band of ~1 kb (Fig. 5B), which was sequenced bidirectionally.

Conserved stretches of the partial shark NCX sequence were aligned with corresponding stretches of other vertebrate NCXs with subsequent cluster analysis (Clustal) to generate an evolutionary tree (Fig. 1B) with consecutive branching: elasmobranch (the new shark sequence) - teleost (trout and tilapia) – amphibian (frog) – mammal (human, dog, cat, rat, mouse). In this comparison the shark sequence deviated more from the mammalian NCX1 sequences than those from frog and bony fish, but more detailed analysis will be required to determine if it can be characterized as NCX1 rather than NCX2 or NCX3, or a novel fish-specific gene. On the other hand the novel shark sequence clearly showed greater homology with known Na^+-Ca^{2+} carrier of solute carrier family 8 (SLC8), than with the K^+-dependent Na^+-Ca^{2+} exchangers, the nearest related family of solute carriers (SLC24).

The shark cardiac NCX was also compared to other vertebrate sequence with respect to the absence or presence of the specific molecular motifs. Some of the observed differences are apparent in the alignment of the aa sequences of the cardiac NCX of dog, frog and shark shown in Fig. 2. Three findings may be relevant to cAMP-mediated regulation:

1) The partial shark sequence followed the cardiac splicing patterns (expressing equivalents of exons ACDEF), but we did not find anything corresponding to the P-loop (GxxxxGKS) coded by exon X of the frog, which so far is the only species known to express this motif in the variable splicing region. Since shark NCX is down-regulated by β-adrenergic stimulation this would suggest that a P-loop in this position is not a strict requirement.

2) At the expected location, the partial shark sequence contains a potential PKA phosphorylation site (RKAAS) conforming to the concensus sequence (R/K)(R/K)xx(S/T) as found in the mammalian NCX1 and NCX3 and frog cardiac NCX, but not in the cardiac NCX of the teleosts trout and tilapia and also not in mammalian NCX2.

3) Unexpectedly, the shark sequence has two conspicuous proline- and alanine-rich inserts at the locations shown in Fig. 1A (10 aa at aa 274, and 54 aa at aa 470, Figs. 1B, 2). These inserts are found at locations where the veterbrate NCX sequences are not highly conserved and even the number of aa's

between adjacent neighboring conserved regions is variable. Such stretches might simply suggest that the aa-strand takes a random turn near the cytoplasmic surface of the protein at an uncritical location, where it has neither important molecular motifs, nor is conserved by steric constraints as it may be in the in the densely packed conserved transmembrane interior of the exchanger. However, such A/P-rich linkers may also represent a functional adaptation since they are thought to provide a high degree of flexibility and may act "as swinging arms that convey... bound intermediates between different sites of a multi-enzyme complex" [1]. Furthermore, searching the draft-genome for fugu (the Japanese puffer fish *Fugu rubripes*, teleost) we find pieces of ~7 NCXs of which one (CAAB01000046) has inserts of 7 and 12 aa (producing at aa 274: HPKEALDGMLEGLEEG and at aa 470: PRVAHRAEVSV LEPNSITSSNSTVGTSSHVPP) at the same locations as in shark NCX. This finding supports the possibility that the isolated shark sequence represents a novel, fish-specific NCX gene.

To sequence the remaining pairts of the N- and C-terminals of the cardiac shark NCX we plan to develop a cDNA library from fresh shark heart mRNA using the ZAP-cDNA GIGAPACK III (Stratagene, Cedar Creek, TX). Cloning into the ZAP II vector will provide exterior sequencing primer sites (T3 and T7 sequences) on the 5' and 3' ends of the gene. Once the sequence for the entire cDNA is known, it can then be cloned into a pGEM expression vector for use in establishing transient and stable cell lines expressing the shark heart NCX1. HEK-293 host cells show no measurable Ni^{2+}-sensitive NCX1 activity prior to infection, and have been used in our previous studies to stably express various NCX1 constructs and examine their cAMP-mediated regulation.

It appears to be a characteristic feature of vertebrate NCXs that considerable variability is achieved by insertions of polypeptide segments at specific locations as result of multiple genes splicing variants. These inserts may provide recognition sequences, binding domains and scaffolding for multi-protein complexes and also a degree of flexibility that facilitates the molecular interactions essential to NCX regulation. Based on physiological experiments with native and mutant cloned NCX molecules and on the rapidly expanding genomic information, that may soon also include elasmobranch species, it will be of interest to ascertain the pattern of NCX adaptations within vertebrate species and determine the modification linked to different environments (osmolarity, ionic strength, temperature) and modes of life, including those that depend on a finely regulated β-adrenergic response. Supported by NIH RO1 HL 16152 + 62525; RMD is the recipient of an American Heart Scientist Development Grant.

1. Cronan J. E., Jr. Interchangeable enzyme modules. Functional replacement of the essential linker of the biotinylated subunit of acetyl-CoA carboxylase with a linker from the lipoylated subunit of pyruvate dehydrogenase. *J. Biol. Chem.* 277: 22520-22527, 2002.
2. Fan J., Shuba Y. M., and Morad M. (1996) Regulation of cardiac sodium-calcium exchanger by beta-adrenergic agonists. *Proc. Natl. Acad. Sci. U S A.* 93: 5527-5532, 1996.
3. He L. P., L. Cleemann, N. M. Soldatov, and M. Morad. Molecular determinants of cAMP-mediated regulation of the Na^+-Ca^{2+} exchanger expressed in human cell lines. *J Physiol.* 548: 677-689, 2003.
4. Nicoll D. A., M. Ottolia, and K. D. Philipson. Toward a topological model of the NCX1 exchanger. *Ann. N. Y. Acad. Sci.* 976: 11-18, 2002.
5. Shuba Y. M., T. Iwata, V. G. Naidenov, M. Oz, K. Sandberg, A. Kraev, E. Carafoli, and M. Morad. A novel molecular determinant for cAMP-dependent regulation of the frog heart Na^+Ca^{2+}xchanger. *J. Biol. Chem.* 273, 18819-18825, 1998.
6. Tong X.-Y., L. Cleemann, and M. Morad. Partial sequencing of the cardiac Na^+-Ca^{2+} exchanger of the spiny dogfish (Squalus acanthias). *Bull. Mt. Desert Isl. Biol. Lab.* 42: 125-126, 2003.
7. Woo S. H., and M. Morad. Bimodal regulation of Na^+-Ca^{2+} exchanger by beta-adrenergic signaling pathway in shark ventricular myocytes. *Proc. Natl. Acad. Sci U S A.* 98, 2023-2028, 2001.