

FULL LENGTH RESEARCH ARTICLE

Cloning, sequence analysis and phylogeny of connexin43 isolated from American black bear heart

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Abstract

Conduction in the heart requires gap junctions. In mammalian ventricular myocytes these consist of connexin43 (Cx43). Hearts of non-hibernating species display conduction disturbances at reduced temperatures. These may exacerbate into lethal arrhythmias. Hibernating species are protected against these arrhythmias by a non-resolved mechanism. To analyze whether the amino acid composition of Cx43 from the hibernating American black bear displays specific features, we cloned the full coding sequence of *Ursus americanus* Cx43 and compared with that of other (non)hibernating species. UaCx43 displays 99.7% identity to rabbit Cx43 at the amino acid level. No specific features were observed in UaCx43 when compared to previously cloned Cx43 from hibernating and non-hibernating mammals. Phylogenetic tree reconstruction of this and other published full-length Cx43 sequences reveals a very high level of conservation from fish to men. Finally, one of the previously identified six mammalian characteristic amino acids, is not conserved in the black bear.

Keywords: *Connexin, heart, phylogeny, hibernation*

Database accession number: DQ833440

Introduction

Connexins are transmembrane proteins that provide intercellular chemical and electrical communication by forming gap junctions (for a detailed review see Sáez et al. (2003)). A hexamer of connexins docks with a similar hexamer on an adjacent cell, thereby forming a pore permeable for ions and small molecules. The connexin gene family is spread throughout vertebrate life forms (Cruciani and Mikalsen 2006) and consists of more than 21 isoforms in mice (Söhl and Willecke 2003). Connexins play important roles in development and normal physiology. Connexin mutations may lead to diseases as congenital deafness, neuropathologies like Charcot-Marie-Tooth disease, skin diseases like erythrokeratoderma variabilis, cataract formation and

oculodentodigital dysplasia (Wei et al. 2004). In the mammalian heart, Cx43 is the main component which permits conduction in the working myocardium (Gros and Jongsma 1996). Failure of normal conduction increases the propensity to life threatening arrhythmias (Kléber and Rudy 2004).

Cooling hearts of rabbits or humans eventually causes conduction block or ventricular arrhythmias (Johansson 1996; Fedorov et al. 2005). The heart of the hibernator contains an intrinsic mechanism to maintain normal conduction patterns at low body temperatures, which prevents arrhythmias during arousal (Van der Heyden and Opthof 2005). In a previous study, we compared the Cx43 amino acid sequences of 18 vertebrate species, including: (i) normothermal mammals, (ii) hibernating mammals

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in which body temperatures can drop well below 5°C, and (iii) species undergoing daily bouts of torpor, in which body temperature drops to 15°C (Van der Heyden et al., 2004). No obvious differences were found between hibernating and non-hibernating species. However, we observed that mammalian Cx43 is characterized by six conserved amino acid positions not present in fish, amphibians and birds. To complete these studies, we now have cloned Cx43 from the hearts of the American black bear. Bears display a unique type of hibernation in which body temperature only drops moderately to reach levels between ~30 and ~35°C (Svihla and Bowman 1954; Hock 1957; Watts et al. 1981 and this study). In cardiac activity, the most prominent change is a substantial decrease in heart rate by 50% or more, pointing to a strong effect on the sinus node. Based on changes in the ECG, additional electrophysiological changes are slowing of atrioventricular conduction and prolongation of the ventricular action potentials

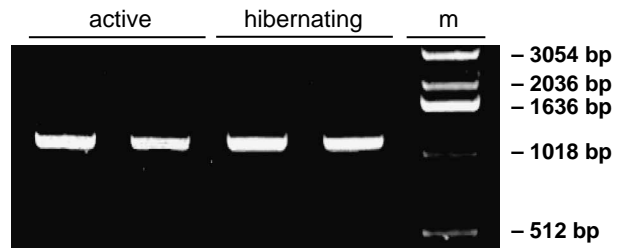


Figure 1. RT-PCR Cx43 products from American black bear from summer active and hibernating animals. Products, obtained in duplo, were of the expected size of approximately 1150 bp. Marker sizes (lane m) are indicated on the right. Samples were run on a 1% agarose, ethidium bromide stained gel.

and, finally, a small decrease of ventricular conduction velocity (Watts et al. 1981; Nelson et al. 2003). Finally, an increase in cardiac contractility has been observed (Nelson et al. 2003). Here we report cloning of black bear Cx43, sequence analysis, species

M G D W S A L G K L L D K V Q A Y S T A	20
ATGGGTGACTGGAGTGCCTTAGGCAAACCTCTTGACAAGGTTCAAGCCTATTCCACTGCT	60
G G K V W L S V L F I F R I L L L G T A	40
GGAGGGAAGGTGTGGCTGTCTGTCTCTTTTCATTTTCCGAATTCTGCTATTGGGGACAGCG	120
V E S A W G D E Q S A F R C N T Q Q P G	60
GTTGAGTCGGCCTGGGTGATGAGCAGTCTGCCTTTTCGTTGTAACACTCAACAACCTGGT	180
C E N V C Y D K S F P I S H V R F W V L	80
TGTGAAAATGTCTGCTACGACAAATCCTTCCCAATCTCTCATGTACGCTTCTGGGTCCTG	240
Q I I F V S V P T L L Y L A H V F Y V M	100
CAGATCATATTTGTGTCTGTTCCACGCTCCTATACCTGGCTCACGTGTTCTACGTGATG	300
R K E E K L N K K E E E L K V A Q T D G	120
CGGAAGGAAGAGAAACTGAACAAGAAAGAGGAGGCTCAAAGTTGCCCAAACAGATGGT	360
V N V E M H L K Q I E I K K F K Y G I E	140
GTCAACGTGGAGATGCACTTGAAGCAGATTGAAATAAAGAAGTTCAAGTATGGTATTGAA	420
E H G K V K M R G G L L R T Y I I S I L	160
GAGCATGGCAAGGTGAAAATGCGAGGGGGCCTGCTGCGAACCTACATCATCAGCATCCTC	480
F K S V F E V A F L L I Q W Y I Y G F S	180
TTCAAGTCTGTCTTCGAGGTGGCCTTCTTGCTGATCCAGTGGTACATCTATGGATTACAGC	540
L S A V Y T C K R D P C P H Q V D C F L	200
TTGAGTGTCTTTTACTACTTGCAAAGAGATCCCTGCCCTCATCAGGTAGACTGCTTCCCTC	600
S R P P T E K T I F I I F M L V V S L V S	220
TCTGCCCCACGGAGAAAACCATCTTCATCATCTTCATGCTGGTAGTGTCTTGGTGTCT	660
L A L N I I E L F Y V F F K G V K D R V	240
CTTGCCCTGAACATCATCGAATCTTCTATGTGTTCTTCAAGGGTGTAAAGGATCGTGTG	720
K G K S D P Y H A T T G P L S P S K D C	260
AAGGGGAAGAGCGATCCTTACCATGCTACCACTGGCCCACTGAGCCCTCCAAAGACTGT	780
G S P P K Y A Y F N G C S S P T A P L S P	280
GGATCTCCGAAATAGCATATTTCAATGGCTGCTCCTCACCCACCGCTCCCTCTCACCC	840
M S P P G Y K L V T G D R N N S S C R N	300
ATGTCTCCTCCTGGGTACAAGCTGGTTACTGGAGACAGAAACAATTCTTCTGCGCAAT	900
Y N K Q A S E Q N W A N Y S A E Q N R M	320
TACAACAACAAGCAAGTGAGCAAACTGGGCTAATTACAGTGCAGAACAAAATCGAATG	960
G Q A G S T I S N S H A Q P F D F P D D	340
GGGCAGGGCGGGAAGCACCATCTCCAACCTCCCATGCACAGCCTTTTGATTTCCCTGATGAC	1020
N Q N S K K L A T G H E L Q P L A I V D	360
AACCAGAAATCTAAAAACTAGCTACTGGGCACGAACCTGCAACCACTAGCCATTGTGGAC	1080
Q R P S S R A S S R A S S R P R P D D L	380
CAGCGGCCTTCCAGCAGAGCCAGCAGCCGTGCCAGCAGCCGACCTCGGCCTGATGACCTG	1140
E I .	382
GAGATATAG	1149

Figure 2. The nucleotide and deduced amino acid sequence of American black bear Cx43. Nucleotide and amino acid (bold) numbers are indicated on the right. Transmembrane regions 1–4 are underlined and bold. Connexin specific extracellular cysteine residues are depicted in white lettering on black background.

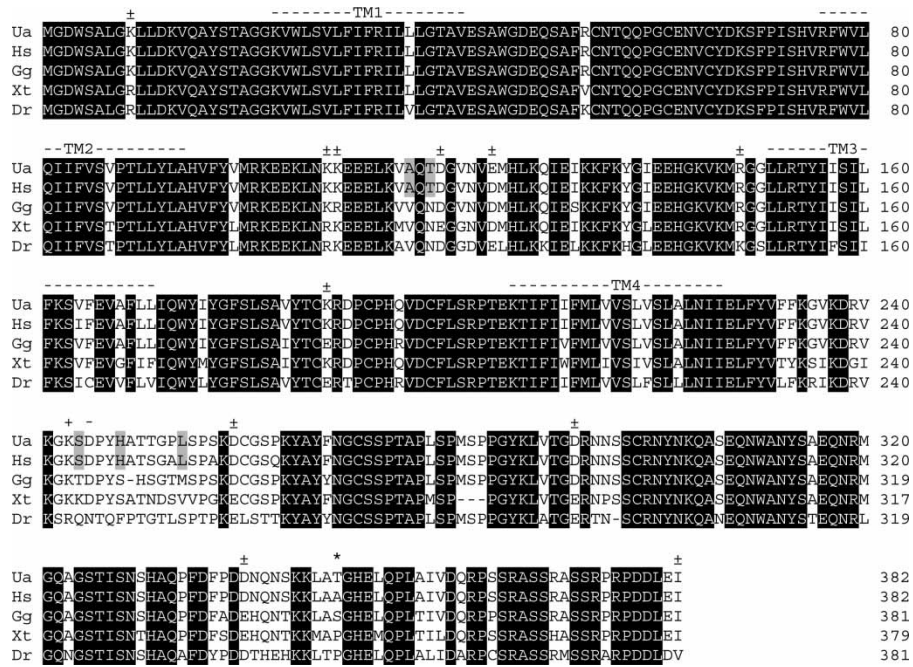


Figure 4. Amino acid alignment of UaCx43 with those of human (Hs), chicken (Gg), western clawed frog (Xt) and zebrafish (Dr). Identical residues in all species are indicated by white lettering on a dark background. Grey shaded, previously denominated as mammalian specific. Amino acid numbers are indicated on the right. Symbols: TM, transmembrane region; ±, conserved charged; + and -, charged residues experimentally confirmed to be involved in gating; * previously denominated as mammalian specific, but not conserved in American black bear.

NM_001002951), MfCx43 (*Macaca fascicularis*, crab-eating macaque AB169817), OmCx43 (*Oncorhynchus mykiss*, rainbow trout; DQ204869) and CuCx43 (*Citellus undulatus*, Siberian ground squirrel; DQ833441). The resulting cladogram (Figure 3) shows clear distinguishable groups (fish, amphibians, birds and mammals) and distinct clades within the mammalian group (old world mice and rat, hamsters, even-toed ungulates, primates). Based on the phylogenetic analysis, American black bear Cx43 is classified into a diverse clade containing dog and hedgehog. The availability of additional Cx43 sequences from other species will further refine the cladogram and improve the internodal statistics in future (Figure 3).

The reliable statistics in the *Muroidea* group, consisting of *Murinae* (old world mice and rats) and *Cricetinae* (hamsters), allows a comparison of the Cx43 substitution rate with that of a summation of four nuclear genes, i.e. GHR, BRCA1, RAG1 and c-Myc, as reported by Steppan et al. (2004). For Cx43 the total branch length for *Phodopus sungorus* from its division from the other hamster species (*Cricetulus griseus* and *Mesocricetus auratus*) is 0.0193 substitutions per site. Steppan et al. found for their summated genes a branch length of ~0.047. Similarly, the branch length of *RnCx43* from its division with *MmCx43* is 0.0259 vs. ~0.038 found by Steppan. Finally, the total branch length separating

Murinae Cx43 from that of *Cricetinae* Cx43 is 0.0400, while Steppan reports ~0.075. Based on these numbers it can be concluded that Cx43 preserves higher levels of conservation than the summation of *GHR*, *BRCA1*, *RAG1* and *c-Myc* genes.

Finally, we compared the UaCx43 amino acid sequence with those human, chicken, *Xenopus* (western-clawed frog) and zebrafish Cx43. Two main regions of dissimilarity are found, located in the intracellular loop and following the fourth transmembrane region, respectively. Many physiological relevant charged amino acids and phosphorylation sites are conserved between the different species (see also Van der Heyden et al. (2004) for a detailed discussion). Furthermore, UaCx43 contains the mammalian specific amino acid residues at position A116, T118, S244, H248 and L254 as defined earlier (Van der Heyden et al. 2004). Remarkably, the sixth recognized mammalian specific amino acid residue, A349, is however not conserved in the American black bear and substituted for threonine. Analysis of other Cx43 from other members of the genus *Ursus* will elucidate whether this is a bear related substitution.

By using a degenerated primer set we thus cloned the first Cx isoform from hearts of the American black bear by an RT-PCR based method. The cloned Cx43 displays all the hallmarks of a genuine Cx43, but is distinct from other mammalian Cx43 identified so far by the A349T substitution.

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