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

MARCEL A. G. VAN DER HEYDEN¹, BART KOK¹, EVELYN N. KOUWENHOVEN¹, OIVIND TOIEN², BRIAN M. BARNES², VADIM G. FEDOROV², IGOR R. EFIMOV³, & TOBIAS OPTHOF¹

¹Department of Medical Physiology, Heart Lung Center Utrecht, University Medical Center Utrecht, Yalelaan 50, 3584 CM Utrecht, The Netherlands, ²Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, AK 99775, USA, and ³Department of Biomedical Engineering, Washington University, St. Louis, MO 63130, USA

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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

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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
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, 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

![Figure 1](image1.png)

**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.
Materials and methods

Experimental animals

Two American black bears (*Ursus americanus*) were captured by biologists of the Alaska Department of Fish and Game, one in the Anchorage vicinity, another in the interior Alaska, and transported by aircraft to Fairbanks, Alaska. The summer bear, an 82.5 kg, male was monitored for health problems in an outdoor enclosure for about 2 weeks before it was immobilized by a UAF veterinarian using Telazol (8–10 mg/kg) administered via pole syringe using a 16 g 1.5 in. needle and brought to the necropsy room on June 30. The hibernating bear, also a male, was kept in a hibernaculum in an undisturbed outdoor enclosure from November 29 to March 10 and then immobilized and transported to the necropsy room with a similar method. The body mass was then 51.2 kg. The core temperature of the undisturbed hibernating bear as recorded with an intraperitoneal temperature transmitter (Mini-mitter Co.) was 34.5°C before immobilization. After transport to the necropsy room the hibernating bear had a rectal temperature of 34.1°C, the summer bear had a rectal temperature of 37.2°C. After clinical blood-sampling from the femoral vein the bear was euthanized with an overdose of pentobarbital. It was then opened for tissue sampling and the heart brought to a −80°C freezer within 15 min.

Cloning of Cx43

Total RNA was isolated from a piece of ventricular heart tissue of both bears using Trizol (Invitrogen, Breda, The Netherlands) according the manufacturers recommendations, and preparations were treated with DNase. cDNA was made using oligo-dT and Superscript II (Invitrogen). Subsequently, Cx43 was amplified by PCR, cloned in pGEM-T-easy (Promega) and sequenced as described previously (Van der Heyden et al. 2004).

Sequence analysis and phylogeny

Alignment and phylogenetic tree reconstruction was performed with MEGA version 3.1 software (Kumar et al. 2004) operating with ClustalW and Neighbor-Joining algorithms. Support for each node was determined by interior-branch test (1000 replicates; seed, 64,238).

Results and discussion

Following RNA isolation from ventricular tissue of the American black bear, cDNA was prepared and the complete Cx43 coding sequence (1146 bp) was amplified in duplo using a degenerated primer combination, of two different animals (Figure 1). Cx43 could be amplified from summer active and hibernating animals, with no obvious differences in amplification product length. Following cloning in pGEM-T-easy vector, Cx43 was sequenced using external T7 and Sp6 primers (Figure 2). The deduced amino acid sequence contains all the hallmarks of a genuine Cx, there are four putative transmembrane regions, three conserved cysteine residues in each extracellularly loop, an intracellular loop between transmembrane regions 2 and 3, and an intracellular located amino and carboxy terminus. At nucleotide level, UaCx43 displays 93.5% identity to cow Cx43. At amino acid level, the percentage of highest identity increases to 99.7% identity with rabbit Cx43.

Next, we investigated the phylogenetic relationships of UaCx43. Therefore, Cx43 sequences were taken from Genbank as previously (Van der Heyden et al. 2004). In addition, four recently deposited Cx43 sequences were added. These are CfCx43 (*Canis familiaris*, dog; accession number FG771145, 2003).

Figure 3. Cladogram of connexin43 sequences. The nucleotide sequences of the protein coding region of 22 Cx43s from fish, avian, amphibians and mammals were analyzed using the ClustalW method and Neighbor-Joining algorithm. Species abbreviations: Bt, *Bos Taurus*; Ca, *Cercopithecus aethiops*; Cc, *Cyprinus carpio*; Cf, *Canis familiaris*; Cg, *Cricetulus griseus*; Cu, *Citellus undulatus*; Da, *Danio rerio*; Dr, *Dianoe aequiplanatus*; Ee, *Erinaceus europaeus*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Ma, *Mesocricetus auratus*; Mf, *Macaca fascicularis*; Mm, *Mus musculus*; Oc, *Oryctolagus cuniculus*; Om, *Onychrophus mykiss*; Ps, *Phodopus sungorus*; Ra, *Rattus norvegicus*; Sc, *Spermophilus citellus*; Ss, *Sus scrofa*; Ua, *Ursus americanus*; Xi, *Xenopus laevis*; Xt, *Xenopus tropicalis*. Species that undergo bouts of hibernation or torpor are boxed. The scale beneath the tree measures the distance between the sequences, and units indicate the number of substitution events. Support for each node (numbers) was determined by interior-branch test, values lower than 50% are not depicted. Catarrhini, humans, great apes, gibbons and old world monkeys; Artiodactyla, even-toed ungulates; Murinae, old world mice and rats; Cricetinae, hamsters.
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 form the other hamster species (Cricetulus griseus and Mesocricetus auratus) is 0.0193 substitutions per site. Steppan et al. found that this sum is 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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References