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Epithelial dominant expression of antifreeze proteins in cunner suggests recent entry into a high freeze-risk ecozone

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ABSTRACT

Most marine teleost fishes residing in a high freeze-risk ecozone, such as the coastal waters of Newfoundland during winter, avoid freezing by secreting high concentrations of antifreeze proteins (AFP) into their blood plasma where they can bind to and prevent the growth of ice that enter the fish. Cunner (*Tautogolabrus adspersus*), which overwinter in such shallow waters are the only known exception. Although this species does produce type I AFP, the plasma levels are too low to be of value as a freeze protectant. Southern and Northern blot analyses carried out in this study establish that the cunner AFP genes belong to a multigene family that is predominantly expressed in external epithelia (skin and gill filaments). These results support the hypothesis that the survival of cunner in icy waters is attributable in part to epithelial AFP that help block ice propagation into their interior milieu. In contrast to the cunner, heterospecifics occupying the same habitat have greater freeze protection because they produce AFP in the liver for export to the plasma as well as in external epithelia. Since the external epithelia would be the first tissue to come into contact with ice it is possible that one of the earliest steps involved in the evolution of freeze resistant fish could have been the expression of AFP in tissues such as the skin. We suggest that this epithelial-dominant AFP expression represents a primitive stage in AFP evolution and propose that cunner began to inhabit "freeze-risk ecozones" more recently than heterospecifics.

1. Introduction

Most marine teleosts are unable to inhabit "freeze-risk ecozones" (subzero ice laden waters) characteristic of polar and sub-polar oceans because the temperature of the water (−1.9 °C) can be a full degree lower than the freezing point of their body fluids (−0.7 to −0.9 °C). A number of teleosts survive in this environment by producing antifreeze proteins (AFP) or glycoproteins (AFGP) that bind to the surface of ice crystals that may form within their body fluids, and thereby inhibit their growth. Without the protective effects of AFP, undercooled fish freeze and die on contact with ice (Scholander et al., 1957; Fletcher et al., 1986).

Three physiologically functional types of AFP (types I–III) as well as the AFGP have been described in a variety of fish taxa (Ewart et al., 1999; Fletcher et al., 2001; Davies et al., 2002; Gauthier et al., 2008). A fourth AFP (type IV) originally identified in longhorn sculpin is no longer considered to function as an antifreeze (Deng et al., 1997; Gauthier et al., 2008). Although diverse in primary sequence and secondary structure, all AFP and AFGP lower the non-equilibrium freezing point of aqueous solutions non-colligatively by binding to specific planes of seed ice, modifying its shape and restricting further growth by an adsorption–inhibition mechanism (Raymond and DeVries, 1977; Knight et al., 1991; Davies et al., 2002; Jia and Davies, 2002).

Type I AFP, the most extensively studied AFP, are alanine-rich, amphipathic α-helical proteins first described in righteye flounders (Duman and DeVries, 1974; Scott et al., 1987; Sicheri and Yang, 1995; Low et al., 2001). These AFP have been fully characterized in four teleost families from three distinct Orders: Order Pleuronectiformes [family Pleuronectidae (Scott et al., 1987, 1988; Knight et al., 1991; Gauthier et al., 2005; Nabeta, 2009)], Order Scorpaeniformes [family Cottidae (Hew et al., 1985; Chakrabartty et al., 1988; Yang et al., 1988; Low et al., 2001) and family Cyclopteridae (Evans and Fletcher, 2001)] and Order Perciformes [family Labridae (Evans and Fletcher, 2004; Hobbs et al., 2011)]. The remarkable similarity in primary sequence and tertiary structure of the AFP from these three Orders of teleosts suggests that they are the result of convergent evolution (Hobbs et al., 2011).

Cunner (*Tautogolabrus adspersus*) belong to a family (Labridae, Order Perciformes) of marine fishes typically found in tropical and subtropical areas (Scott and Scott, 1988). However this species is distributed along the Atlantic coast of North America from the temperate waters of Chesapeake Bay to the north coast of Newfoundland Canada, where freezing water temperatures and ice during the winter cause a significant threat to their survival (Green, 1974). Although AFP present

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in cunner are believed to play a role in their ability to survive such harsh conditions (Valerio et al., 1990; Evans and Fletcher, 2004) a recent study has demonstrated that the plasma levels are too low to significantly improve their freeze resistance (Hobbs et al., 2011). Thus the physiological significance of AFP in cunner plasma remains in question.

Previous research has shown that cunner skin, in contrast to blood plasma, has relatively high levels of AFP activity leading to the suggestion that the AFP could serve to help prevent ice propagation across external epithelial tissues (Valerio et al., 1990). Such a protective effect has been demonstrated for winter flounder where AFP have been shown to help prevent ice propagation across the skin (Valerio et al., 1992a). This skin protective hypothesis is supported at the molecular level by the discovery of a novel subclass of type I AFP genes (skin-type) that are predominantly expressed in the epithelial tissues of the winter flounder (*Pseudopleuronectes americanus*) and in two species of sculpin, shorthorn (*Myoxocephalus scorpius*) and longhorn (*Myoxocephalus octodecemspinosus*).

Our recent sequencing of a cunner cDNA library indicates that the cunner AFP gene codes for a protein that lacks an obvious secretary signal sequence; a characteristic that is shared by all of the skin-type I AFP. However since the library used to isolate the cunner AFP cDNA was derived from a mixture of liver and skin mRNA the tissue source of the AFP mRNA is unknown.

The present study establishes that cunner AFP belong to a multigene family that is predominantly expressed by the external epithelia. In addition, the cunner's closest relative (*Tautoga onitis*) on the western Atlantic and a resident of more southerly waters also possessed a multigene family of type I AFP genes. It is suggested that cunner began to inhabit "freeze risk ecozones" more recently than the heterospecifics.

2. Materials and methods

2.1. Sample Collection

Cunner (*T. adspersus*) were collected by Ocean Sciences Centre (OSC) divers near Logy Bay, Newfoundland, Canada in the winter of 2008 and maintained at the OSC in 250 L aquaria supplied with flowing seawater (32–33‰) under seasonally normal photoperiod and water temperatures. Water temperatures ranged from 15.1 °C in August to −1.8 °C during April. Fish were euthanized with an MS-222 overdose and the tissues were removed immediately, frozen in liquid nitrogen and stored at −70 °C.

Tautog (*T. onitis*) were collected by otter trawl near Menemsha Bight, Martha's Vineyard, MA, USA and maintained at the Marine Biological Laboratory, Woods Hole, MA, USA in a 20,000 L aquarium supplied with running seawater. Fish were killed with MS-222 overdose and liver samples were excised, cut into ~1 mm thick pieces and treated with DMSO/salt DNA preservation solution (20% DMSO, 0.25 M sodium EDTA, saturated NaCl, pH 7.5) as previously described (Seutin et al., 1991).

2.2. RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE)

Total RNA was isolated from the cunner tissue samples using TRIzol® reagent (Invitrogen) as per the manufacturer's instructions. GeneRacer™ cDNA was generated from total RNA by ligation of the GeneRacer™ RNA oligo to full length mRNA followed by reverse transcription with the GeneRacer™ poly dT primer and superscript III reverse transcriptase as per the manufacturer's instructions (Invitrogen). GeneRacer™ cDNA was used as the template for primary PCR with GeneRacer™ 3′/Cunner F1 or GeneRacer™ 5′/Cunner R1 primers (Fig. 1). This was followed by nested PCR using the primary PCR product diluted 1/20 in water as the template with GeneRacer™ 3′ nested/Cunner F2 or GeneRacer™ 5′ nested/Cunner R2 primers to amplify both the 5′ and 3′ ends of the cunner AFP gene. The nucleotide sequences of the primers used (in 5′–3′ direction) were:

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1   cagattttgtggatcaagttcaatagttctctctctacaacaaaATGGAT
                  PF1
51  TCAGGAAAAAGTGCACCTCAGCTTGCTGCTGAAGCTACTGCTGCAGCTGC
        Cunner F1       Cunner F2
101 ACTTAAAGCTGCTGAAGCTACCAAAGCTGCAGCTAAAGCTGCAGCTGAAG

151 CTACTGCTAAAGCTGCAGCTGCAGCTGCTGGAAAATAGctgctggagctg

201 caggggaagccgctgcttcgtcctttgggccaactaaaatggctgctggc
                        Cunner R2       Cunner R1
251 ccagggatttttccagctatttggaggatgtgcacactgtctaacttgaa

301 atggacttcattgccaacgaatgtatgtctgcaagcatacatgagttagc

351 atgctattttctatgtacttaaaatcatgactttgcacatcttttaccta
                                                PR1
401 cgggtacaaataaagtaacctgaaagtttgaaactgtcaaaaaaaaaa
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Fig. 1. Nucleotide sequence of the complete *Tautogolabrus adspersus* liver/skin cDNA generated by RLM-RACE illustrating primer annealing sites. The ORF is capitalized, whereas the 5′- and 3′-UTRs are in lower case letters. In frame start and stop codons are in bold. Primers PF1, Cunner F1, Cunner R1 and PR1 are underlined while Cunner F2 and Cunner R2 are italicized.

Cunner F1: GAT TCA GGA AAA AGT GCA CCT CAG CTT
Cunner R1: CTG GGC CAG CAG CCA TTT TAG TTG G
Cunner F2: CAC CTC AGC TTG CTG CTG AAG CTA C
Cunner R2: TTA GTT GGC CCA AAG GAC GAA GCA GC
PF1: GTG GAT CAA GTT CAA TAG TTC TC
PR1: TGT ACC CGT AGG TAA AAG ATG TG

PCR reactions were performed in a Mastercycler® gradient thermo cycler (Eppendorf Scientific). Amplification was achieved in a 25 μL reaction containing one unit of Accuprime™ GC Rich DNA polymerase (Invitrogen), 1×AccuPrime™ GC-rich buffer B [60 mM Tris–HCl (pH 9.2), 1.5 mM $MgSO_4$, four dNTP's (200 μM each of dATP, dGTP, dCTP and dTTP), 30 mM NaCl with proprietary thermostable AccuPrime™ proteins and enhancers], template cDNA (100 ng for primary PCR and 2 μL of the diluted primary PCR product for nested PCR) and 0.2 μM of both 5′ and 3′ primers. The conditions for PCR were as follows: initial denaturation at 95 °C for three min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s and elongation at 72 °C for 90 s. A final step at 72 °C for 10 min ensured complete elongation of products. PCR products were separated on 1.2% agarose/TBE (45 mM Tris-borate, 1 mM EDTA) gels containing 0.5 μg/mL ethidium bromide for nucleic acid visualization.

2.3. Examination of tissue specific expression of the cunner AFP gene

2.3.1. RNA probe production

2.3.1.1. Bacterial preparation. Cunner AFP cDNA was amplified with PCR using primers PF1/PR1 and a cunner GeneRacer™ skin/liver cDNA pool as the template. Cycling conditions were as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 54 °C for 30 s and elongation at 72 °C for 90 s. A final step at 72 °C for 10 min ensured complete elongation of products. The resulting PCR product was separated on 1.2% agarose/TBE gels containing 0.5 μg/mL ethidium bromide and extracted using the QIAquick gel extraction kit (Qiagen) as per the manufacturer's instructions. The purified cDNA was ligated into the pGEM®-T easy vector and transformed into *Escherichia coli* bacteria [DH5α (Invitrogen)] according to the pGEM®-T easy vector system protocol (Promega).

2.3.1.2. Plasmid purification. Bacteria were grown in Luria–Bertani (LB) medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, pH 7.0) which was supplemented with ampicillin (100 μg/mL)