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Hypoxia-Inducible Factor-1 α in Antarctic notothenioids contains a polyglutamine and glutamic acid insert that varies in length with phylogeny

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Abstract

The long evolution of the Antarctic perciform suborder Notothenioidei in the icy, oxygen-rich waters of the Southern Ocean may have reduced selective pressure to maintain a hypoxic response. To test this hypothesis, cDNA of the key transcriptional regulator of hypoxic genes, hypoxia-inducible factor-1 α (HIF-1 α), was sequenced in heart ventricles of the red-blooded notothenioid, *Notothenia coriiceps*, and the hemoglobinless icefish, *Chaenocephalus aceratus*. HIF-1 α cDNA is 4500 base pairs (bp) long and encodes 755 amino acids in *N. coriiceps*, and in *C. aceratus*, HIF-1 α is 3576 bp long and encodes 779 amino acids. All functional domains of HIF-1 α are highly conserved compared to other teleosts, but HIF-1 α contains a polyglutamine/glutamic acid (polyQ/E) insert 9 amino acids long in *N. coriiceps* and 34 amino acids long in *C. aceratus*. Sequencing of this region in four additional species, representing three families of notothenioids, revealed that the length of the polyQ/E insert varies with phylogeny. Icefishes, the crown family of notothenioids, contain the longest polyQ/E inserts, ranging between 16 and 34 amino acids long, whereas the basal, cold-temperate notothenioid, *Eleginops maclovinus*, contains a polyQ/E insert only 4 amino acids long. PolyQ/E inserts may affect dimerization of HIF-1 α and HIF-1 β , HIF-1 translocation into the nucleus and/or DNA binding.

Keywords

Hypoxia; hypoxia-inducible factor-1; notothenioids; icefishes

INTRODUCTION

Members of the Antarctic perciform suborder Notothenioidei, which dominate the fish fauna on the continental slope and shelf surrounding Antarctica, are highly stenothermic (Somero and DeVries 1967). The ancestral notothenioid survived cooling of the Southern Ocean that began 38 million years ago and resulted in a precipitous decline in species abundance and diversity in the mid-tertiary period (Eastman 1993). Water temperatures have been less than 5 °C for approximately 12 million years and today hover around –1.87 °C year round (Eastman 1993; Eastman 2005). While the long evolution of notothenioids in an oxygen-rich

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and thermally stable, cold environment has equipped them with a myriad of traits enhancing their fitness in the cold, including antifreeze glycoproteins, cold-stable microtubules, membranes enriched in polyunsaturated fatty acids, and high expression of genes that mitigate cold-stress, it has also resulted in tradeoffs, potentially reducing their capacity to endure changes in the environment (DeVries and Wohlschlag 1969; Detrich et al. 1987; Römisch et al. 2003; Chen et al. 2008). In general, the transcriptional response to heat stress is muted in Antarctic fishes compared to temperate ones (Podrabsky 2004; Bilyk and Cheng 2014; Huth and Place 2016). Notably absent is a robust heat shock response and upregulation of heat shock proteins (molecular chaperones) in response to heat stress (Hofmann et al. 2000; Huth and Place 2013; Bilyk and Cheng 2014). In fact, exposure of *Pagothenia borchgrevinki* to 4 °C for 2–4 days (Bilyk and Cheng 2014) and *Trematomus bernacchii* to 4 °C for 28 days (Huth and Place 2013), resulted in a decrease in levels of Hsp mRNAs. Upregulation of HSP90 has been observed in response to a combined heat and pCO₂ stress of *P. borchgrevinki* (Huth and Place 2016), and of HSP70 in blood of *Notothenia coriiceps* held at 4 °C for 48 hrs (Shin et al. 2014). However, no studies to date have observed an increase in any of the Hsp proteins in notothenioids in response to heat stress. The capacity of notothenioids to withstand hypoxia, which often coincides with warming, has not been thoroughly investigated.

The transcription factor hypoxia-inducible factor-1 (HIF-1) is critical for maintaining cellular function during hypoxia. HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β subunits (Semenza et al. 1997). HIF-1 β , also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed, whereas HIF-1 α levels are regulated by the partial pressure of oxygen (Ivan et al. 2001; Semenza 2004). Under normoxia, HIF-1 α is hydroxylated at two proline residues in an oxygen-dependent reaction catalyzed by prolyl hydroxylase, which targets HIF-1 α for ubiquitination and degradation by the proteasome (Jaakkola et al. 2001; Min et al. 2002; Ely et al. 2014). Under hypoxic conditions, HIF-1 α is stabilized, enters the nucleus, dimerizes with HIF-1 β , and along with the co-activators, CREB-binding protein (CBP) and p300, transactivates the expression of hundreds of genes, including ones involved in the growth of blood vessels, glycolysis, cell growth and survival, and erythropoiesis (Semenza et al. 1997; Jiang et al. 1997; Berta et al. 2004; Hong et al. 2004; Semenza 2004).

We hypothesized that the long evolution of notothenioids in the cold stable, oxygen-rich waters of the Southern Ocean might have reduced selective pressure on the hypoxia response pathway. To address this hypothesis, we sequenced the cDNA of HIF-1 α from heart ventricles of the red-blooded notothenioid *N. coriiceps* and a member of the hemoglobinless family of Channichthyidae, *Chaenocephalus aceratus*. Sequences revealed a polyglutamine/glutamic acid (polyQ/E) insert that varied in length between the two species, and so to determine the prevalence of this insert, we obtained partial sequence of HIF-1 α cDNA from hearts of *Eleginops maclovinus*, *Chionodraco rastrospinosus*, *Parachaenichthys charcoti*, and *Gymnodraco acuticeps*.

MATERIALS AND METHODS

Chaenocephalus aceratus (Lönnerberg 1906), *Chionodraco rastrospinosus* (Dewitt and Hureau 1979), and *Notothenia coriiceps* (Richardson 1844) were collected in Dallmann Bay, Antarctica (64°10'S, 62°35'W) by benthic trawl or with baited traps deployed from the ARSV Laurence M. Gould (LMG) during the austral fall of 2011. *Parachaenichthys charcoti* (Vaillant 1906) and *Gymnodraco acuticeps* (Boulenger 1902) were collected during the austral fall of 2005. Animals were held in circulating seawater tanks at 0 ± 0.5 °C on board the LMG and then transferred to aquaria at the U.S. Antarctic research station, Palmer Station, where they were maintained in circulating seawater tanks at 0 ± 0.5 °C. Animals were killed by a sharp blow to the head followed by transection of the spinal cord. Ventricles were quickly excised, allowed to contract while submerged in Notothenioid Ringer's solution (260 mM NaCl, 2.5 mM MgCl₂, 5 mM KCl, 2.5 mM NaHCO₃, 5 mM NaH₂PO₄, pH 8.0). The atria and aorta were removed, and the ventricle cut into small pieces, frozen in liquid nitrogen, and stored at -80 °C or immersed in RNAlater Solution (Ambion, Thermo Fisher Scientific, Wilmington, DE) overnight at 4 °C and then frozen at -80 °C. Samples were stored at -80 °C until use. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (134774-2). Samples of *Eleginops maclovinus* (Cuvier 1830) were provided by Dr. Christina Cheng, stored in 90% ethanol.

RNA was extracted using the RNeasy mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). RNA was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific) and only samples with a 260/280 ratio >2 and 260/230 >1.6 were used in the subsequent steps. RNA integrity was determined by agarose gel electrophoresis.

The cDNA was prepared for sequencing from two individuals each of *C. aceratus* and *N. coriiceps* using Clontech SMARTer RACE 5'/3' kit (Takara Bio USA, Mountain View, CA) following the manufacturer's protocol. Gene-specific primers were designed from previously published partial HIF-1 α sequences from *N. coriiceps* and *C. aceratus* (GenBank accession nos. GU362089 and GU362090, respectively) using Primer-BLAST (National Center for Biotechnology Information (NCBI), Bethesda MD) (5' RACE and 3' RACE in Table 1). DNA was isolated from four clones of each individual. Bacteria were grown overnight at 37 °C in 2 mL LB broth supplemented with 2 μ L of 100 mg mL⁻¹ ampicillin. Plasmid DNA was isolated using the QIAprep Miniprep Kit (Qiagen) and quantified using a NanoDrop ND-1000. The length of the cDNA inserts was verified by digesting plasmid DNA with EcoRI and HindIII (Invitrogen, Thermo Fisher Scientific). The cDNA was labeled for sequencing using Big Dye (Applied Biosystems, Thermo Fisher Scientific) with an iCycler (Bio-Rad, Hercules, CA) or done by GeneWiz (GeneWiz, South Plainfield, NJ). Reactions were cleaned-up using Sephadex G-50 illustra (GE Healthcare, Pittsburg, PA) before sequencing by GeneWiz using Big Dye 3.1 and 373 sequencers. Sequencing was performed using the primers M13F (-41), M13R, 5' Seq and 3' Seq, 5' gap and 3' gap (Table 1). All but the M13F (-41) and M13R were designed using Primer-BLAST. GeneWiz provided the primers M13F (-41) and M13R.

The cDNA region containing the glutamic acid (E) and glutamine (Q) insert was sequenced from heart ventricles of *C. aceratus* (n=2, in addition to the two individuals used to obtain

full-length sequences), *C. rastrospinosus* (n=5), *P. charcoti* (n=3), *G. acuticeps* (n=3) and *E. maclovinus* (n=2) using direct sequencing and primers designed with Primer-BLAST (5' polyQ and 3' polyQ in Table 1). GeneWiz used proprietary techniques to determine the length of the highly repetitive region.

Sequences were aligned with Genome Compiler version 2.2.86 and consensus sequences were generated for each species (Genome Compiler Corporation, Los Altos, CA). Only sequences with a read length >400 bp and a QC score >30 were used. Discrepancies with the published sequence were resolved by sequencing additional clones. Sequences were created based on majority consensus. Amino acid sequences were determined using both Serial Cloner version 2.5 and BLAST (SerialBasics, France and NCBI). Conserved domains were detected using BLAST and the complete sequences were aligned with *Gasterosteus aculeatus* using Clustal Omega (submitted via <http://www.ebi.ac.uk/Tools/msa/clustalo/>).

The ratio of non-synonymous to synonymous mutations (dN/dS) was calculated using HIF-1 α cDNA or predicted cDNA sequences from *C. aceratus* (GenBank accession no. KX950828), *N. coriiceps* (GenBank accession no. KX950829), *Oncorhynchus mykiss* (GenBank accession no. NM_001124288.1), *Takifugu rubripes* (GenBank accession no. XM_003962474.2), *Danio rerio* (GenBank accession no. AY326951.1), *Gasterosteus aculeatus* (GenBank accession no. NM_001267649.1), and *Homo sapiens* (GenBank accession no. NM_001530.3). Sequences were aligned using ClustalW (codons) algorithm in MEGA7 (Kumar et al. 2016) with a gap opening penalty of 15. A species tree was generated in MEGA7 by creating a Maximum Likelihood Tree with a Tamura-Nei model (Tamura and Nei 1993). The dN/dS ratio was calculated using Codeml in the Phylogenetic Analysis by Maximum Likelihood (PAML), version 4.9 (Yang 2007). The following settings were used for the Codeml branch model: seqtype = 1, CodonFreq = 2, model = 2, NSsites = 0, and fix_omega = 0 and clean data = 0. PAML interprets sequence gaps, such as the polyQ/E coding region, as ambiguous so only *N. coriiceps*, having the shortest polyQ/E insert among notothenioids, was included in the analysis.

RESULTS

The full-length cDNA sequence of HIF-1 α in *N. coriiceps* is 4500 bp with an open reading frame that encodes 755 amino acids (Fig. 1). In *C. aceratus*, HIF-1 α cDNA is 3576 bp with an open reading frame that encodes 779 amino acids (Fig. 1). Complete cDNA sequences are available in GenBank (KX950828 for *C. aceratus* and KX950829 for *N. coriiceps*). The amino acid sequence of HIF-1 α from *C. aceratus* is 95% identical to that of *N. coriiceps* and 78% identical to *G. aculeatus*, while the amino acid sequence of HIF-1 α from *N. coriiceps* is 79% identical to *G. aculeatus*. Most notable, *N. coriiceps* possesses a polyQ/E insert that is 9 amino acids long, whereas this insert is 34 amino acids long in *C. aceratus* (Fig. 1).

The HIF-1 α protein contains a basic Helix Loop Helix (bHLH) domain for DNA binding, Per Arnt Sim (PAS) domains for heterodimerization, the HIF-1 domain that binds regulatory proteins, a HIF-1 α C-terminal transactivation domain (CTAD) that is important for interactions with other transcription factors, two proline residues that are hydroxylated under normoxic conditions, and two nuclear localization signals (NLS) (Semenza et al. 1997;

Lando et al. 2002; Hon et al. 2002; Sollid et al. 2006). These functional domains are highly conserved in notothenioids (Fig. 1). Compared to HIF-1 α in humans (GenBank accession no. NP_001521.1) the degree of conservation of functional groups is as follows: 96% for the NLS, 85% for the bHLH, 79% for the PAS domains, 72% for HIF-1, and 68% in HIF-1 α CTAD for both *C. aceratus* and *N. coriiceps*; the proline residues are 100% conserved in *C. aceratus* and 92% conserved in *N. coriiceps*. Compared to stickleback, the functional domains are also highly conserved, as follows: 91% for NLS, 93% for bHLH, 92% for PAS, 100% for HIF-1, and 84% for HIF-1 α CTAD in both *C. aceratus* and *N. coriiceps*; the proline residues are 100% conserved in *C. aceratus* and 92% conserved in *N. coriiceps*. Between *N. coriiceps* and *C. aceratus*, conservation of the amino acids in the functional domains is 100% except for the PAS domains (98% identical) and the proline residues (92% identical).

To further investigate the prevalence of the polyQ/E repeats in notothenioids, HIF-1 α cDNA was sequenced in four additional notothenioids, representing the Bathydraconidae (*G. acuticeps* and *P. charcoti*), Channichthyidae (*C. rastrospinosus*), and Nototheniidae (*E. maclovinus*) families. The HIF-1 α cDNA lengths obtained ranged from 827 bp to 877 bp and aligned to 896 bp to 1717 bp of the full-length cDNA for HIF-1 α in *N. coriiceps*. The length of the polyQ/E insert reflects phylogeny and is longest in the icefishes (*C. rastrospinosus*= 25 amino acids), intermediate in the bathydraconids (*P. charcoti*= 16 amino acids, *G. acuticeps*= 9 amino acids), and shortest in the cold-temperate notothenioid, *E. maclovinus* (4 amino acids) (Figs. 2 and 3).

The dN/dS ratio is higher in fishes than humans, and higher in *N. coriiceps* and temperate fish species (*G. aculeatus* and *O. mykiss*) compared to tropical species (*T. rubripes* and *D. rerio*) (Fig. 4).

There was some variation in the length of the polyQ/E insert in HIF-1 α within bathydraconids and icefishes. One of the five individuals of *C. rastrospinosus* sequenced had an insert four amino acids shorter than the others, while one individual had an insert that was one amino acid longer than the others. One of the four *C. aceratus* sequenced had an insert that was two amino acids longer than the others. Within two of *C. aceratus*, for which multiple clones were sequenced, the length of the insert varied between 32 and 38 amino acids. The previously published sequence for *C. aceratus* (GU362090) contained a polyQ/E insert 38 amino acids long. One of the three *G. acuticeps* sequenced had two additional Qs in the insert compared to the others. One of the three *P. charcoti* sequenced had an insert that was two amino acids shorter than the others. These differences in the length of the polyQ/E insert may be due to the presence of multiple isoforms and/or sequencing errors. However, GeneWiz employs a proprietary method for effectively sequencing tri-nucleotide repeats, suggesting it is more likely natural variation among and within individuals.

DISCUSSION

The functional domains of HIF-1 α are highly conserved in notothenioids, yet HIF-1 α contains a polyQ/E insert in notothenioids that varies with phylogeny. The longest insert is in *C. aceratus* (34 amino acids) and the shortest is in the cold-temperate notothenioid, *E.*

maclovinus (4 amino acids). The polyQ/E insert in *E. maclovinus* is similar in size to that of the temperate teleost, *G. aculeatus* (threespine stickleback), which has two E's followed by two leucines and two Q's in this region (Fig. 2). Some mammals, including *Homo sapiens* (NP_001521.1), *Canis lupus familiaris* (NP_001274092.1), and *Bos taurus* (BAA78675.1) have a single E, while others, such as *Mus musculus* (CAA70306.1) have no E or Q residues in this region (corresponding to amino acids 363–397 in *N. coriiceps*).

The HIF-1 α cDNA of the Antarctic notothenioid, *N. coriiceps*, displays a higher dN/dS ratio compared most teleosts (except *O. mykiss*), suggesting relaxed purifying selection operating on HIF-1 α in Antarctic notothenioids, perhaps due to their cold, stable environment.

Consistent with this, a comparison of HIF-1 α DNA sequence among teleosts found the highest dN/dS ratios in teleosts inhabiting cold environments (Cao et al. 2008). Also, whole genome sequencing of *N. coriiceps* found higher dN/dS ratios in genes of *N. coriiceps* compared to temperate fishes (Shin et al. 2014). One caveat of the dN/dS calculation is that it does not include the polyQ/E region of HIF-1 α because is not conserved among species and therefore interpreted as ambiguous in the analysis.

Protein modeling using PredictProtein (www.predictprotein.org) (Rost et al. 2004; Yachdav et al. 2014) places the polyQ/E region of HIF-1 α exposed on the surface of the protein where it may be involved in protein-protein interactions. In stickleback, the region surrounding the polyQ/E insert (amino acids 363 – 382) is only partially exposed and forms a helix, which is absent in HIF-1 α of Antarctic notothenioids. Thus, the polyQ/E insert appears to disrupt formation of the helix and increase the surface area of the protein.

The functions of polyQ regions are fairly well characterized, as 0.34% of the human proteome contains polyQ regions (Schaefer et al. 2012). Although notothenioid HIF-1 α contains a polyQ/E repeat rather than a polyQ repeat, the functional implications may be similar. Both Q and E are polar amino acids; their structure differs only in that Q possesses an additional amino group. PolyQ regions are associated with protein aggregation and implicated in several human diseases, such as Huntington's, in which polyQ repeats are 10 to 34 amino acids long in healthy individuals but over 36 amino acids long in those with the disease (Duyao et al. 1993). Moreover, increases in the number of polyQ repeats leads to earlier onset of the disease and a more severe phenotype (Scherzinger et al. 1997). In addition to aggregation, polyQ regions may cause protein misfolding, altering protein function and interactions with other proteins (Zoghbi and Orr 2000; Gidalevitz et al. 2006). PolyQ repeats also create RNA hairpins and sequester RNA processing proteins making the mRNA difficult to translate and result in decreased protein production (Nalavade et al. 2013). Consistent with this, HIF-1 α mRNA levels are 6.6-fold lower in hearts of *C. aceratus* compared to *N. coriiceps* at ambient temperature (Devor et al. 2016).

Although polyQ regions are often linked to disease, the prevalence of polyQ repeats in proteins suggests they also possess important biological functions. The most common functions of proteins with polyQ regions are DNA and protein binding, and include transcriptional regulation and chromatin maintenance (Schaefer et al. 2012). In support of their beneficial effects, transcription factors with 10–34 length polyQ regions enhance transcriptional activity in-vitro (Gerber et al. 1994). Therefore, it is conceivable that the

longer polyQ/E repeat in hemoglobinless icefishes enhances DNA binding and activity of HIF-1, thereby bolstering their response to hypoxia. Alternatively, the extensive polyQ/E repeat in icefishes may impair HIF-1 function and could explain their lower thermal tolerance compared to the red-blooded species (Beers and Sidell 2011). The lower blood oxygen-carrying capacity of hemoglobinless icefishes does not explain their lower thermal tolerance compared to red-blooded species, as addition of supplemental oxygen does not increase their critical thermal maximum (CTMax) (Devor et al. 2016). Rather, our recent studies (unpublished Egginton, Farrell, O'Brien and Crockett) indicate that similar to temperate fishes (Clark et al. 2008; Eliason et al. 2011; Anttila et al. 2013; Sandblom et al. 2016), cardiac performance may limit thermal tolerance. The series circulation and lack of a cardiac coronary circulation in notothenioids may make their hearts especially vulnerable to hypoxia during warming (Davie and Farrell 1991). Moreover, hearts of *C. aceratus* lack the intracellular binding protein, myoglobin, potentially further exacerbating hypoxic conditions (Sidell et al. 1997). These traits, coupled with a potentially non-functional HIF-1, may contribute to the low thermal tolerance of icefishes. Consistent with this, knocking out HIF-1 α in *Caenorhabditis elegans* reduces heat tolerance and survivorship (Treinin et al. 2003). Current studies in our lab are aimed at elucidating the functional implications of the polyQ/E insertions on HIF-1 α function in notothenioids.

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conserved, (:) is strongly conserved, scoring >0.5 in the Gonnet PAM 250 matrix, (.) is weakly conserved, scoring ≤ 0.5 in the Gonnet PAM 250 matrix, and (–) is not conserved.

[illegible]

Fig. 2.
Amino acid alignment of the polyQ/E insert region of HIF-1 α of *G. aculeatus* (NP_001254578), *E. maclovinus* (KX950830), *N. coriiceps* (KX950829), *P. charcoti* (KX950833), *G. acuticeps* (KX950832), *C. rastropinosus* (KX950831), and *C. aceratus* (KX950828) with conservation scoring. The sequence aligns to amino acids 319 to 515 of HIF-1 α in *N. coriiceps*. Amino acid conservation scoring was determined using Clustal Omega (*): fully conserved, (:): strongly conserved, scoring >0.5 in the Gonnet PAM 250 matrix, (.) is weakly conserved, scoring 0.5 in the Gonnet PAM 250 matrix, and (–) is not conserved. The PAS domains and polyQ/E insert are boxed and labeled. One of the two proline recognition sequences is enclosed in a rounded box.

Fig. 3.

Phylogeny of notothenioids with the length of their polyQ/ E insert in parentheses. Species names in bold were determined by this study; others were determined using sequences in GenBank as follows: KX950830 for *E. maclovinus*, DQ089694 for *T. pennellii*, DQ089682 for *T. hansonii*, GU362091 for *G. gibberifrons*, KX950829 for *N. coriiceps*, KX950832 for *G. acuticeps*, KX950833 for *P. charcoti*, KX950831 for *C. rastrospinosus*, and KX950828 for *C. aceratus*. Phylogenetic tree was created based on trees published by Near et al. (2012).

Fig. 4.

The dN/dS ratio for several species of teleosts and humans. The tree was created using MEGA7 and dN/dS ratios were obtained from CODEML in PAML. Additional sequences used to create the tree were obtained from GenBank: *Oncorhynchus mykiss* (NM_001124288.1), *Takifugu rubripes* (XM_003962474.2), *Danio rerio* (AY326951.1), *Gasterosteus aculeatus* (NM_001267649.1), and *Homo sapiens* (NM_001530.3).

Table 1

Primer sequences used for generating and sequencing HIF-1 α cDNA

Primer Name	Primer Sequence
5' RACE	5'-GATTACGCCAAGCTTATGGTGCTGACATGGAGGGGCTCGCACG-3'
3' RACE	5'-GATTACGCCAAGCTTCACTCAGGCGGCAGAGGCCAGCCGTTT-3'
5' Seq	5'-AAGGCAGGTCGTGGAAGTGG-3'
3' Seq	5'-TAGGTAGGAGCCGTTGAGCT-3'
5' gap	5'-GACATCCACAGCTATCCCTTC-3'
3' gap	5'-AGCTTACAATACACTGCTGGC-3'
5' polyQ	5'-GGACACCAAGACATTCTCAGC-3'
3' polyQ	5'-ATCAATGGCAAACAGCTTCTCC-3'
M13R	5'-CAGGAAACAGCTATGAC-3'
M13F(-41)	5'-GGTTTTCCAGTCACGAC-3'