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bloodthirsty, an RBCC/TRIM gene required for erythropoiesis in zebrafish

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Abstract

The Antarctic icefishes (family Channichthyidae, suborder Notothenioidei) constitute the only vertebrate taxon that fails to produce red blood cells. These fishes can be paired with closely related, but erythrocyte-producing, notothenioids to discover erythropoietic genes via representational difference analysis. Using a B30.2-domain-encoding DNA probe so derived from the hematopoietic kidney (pronephros) of a red-blooded Antarctic rockcod, *Notothenia coriiceps*, we discovered a related, novel gene, *bloodthirsty* (*bty*), that encoded a 547-residue protein that contains sequential RING finger, B Box, coiled-coil, and B30.2 domains. *bty* mRNA was expressed by the pronephric kidney of *N. coriiceps* at a steady-state level 10-fold greater than that found in the kidney of the icefish *Chaenocephalus aceratus*. To test the function of *bty*, we cloned the orthologous zebrafish gene from a kidney cDNA library. Whole-mount in situ hybridization of zebrafish embryos showed that *bty* mRNA was present throughout development and, after the mid-blastula transition, was expressed in the head and in or near the site of primitive erythropoiesis in the tail just prior to red cell production. One- to four-cell embryos injected with two distinct antisense morpholino oligonucleotides (MOs) targeted to the 5′-end of the *bty* mRNA failed to develop red cells, whereas embryos injected with 4- and 5-bp mismatch control MOs produced wild-type quantities of erythrocytes. The morphant phenotype was rescued by co-injection of synthetic *bty* mRNA containing an artificial 5′-untranslated region (UTR) with the antisense MO that bound the 5′-UTR of the wild-type *bty* transcript. Furthermore, the expression of genes that mark terminal erythroid differentiation was greatly reduced in the antisense-MO-treated embryos. We conclude that *bty* is likely to play a role in differentiation of the committed red cell progenitor.
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Keywords: *Chaenocephalus aceratus*; *Notothenia coriiceps*; *Danio rerio*; B30.2; *bloodthirsty* (*bty*) gene; Icefish; Rockcod; Zebrafish; Erythropoiesis; Hematopoiesis; RING-B box-coiled-coil (RBCC); Tripartite motif (TRIM)

Introduction

Fishes constitute approximately 50% of all vertebrate species and are attractive organisms for analysis of hematopoiesis. Lacking bone marrow, teleost fishes form blood cells in three tissues: the pronephric or head kidney, the spleen, and the thymus (Rowley et al., 1988). Although the site of erythropoiesis (pronephros and/or spleen) varies, teleosts express both the primitive and definitive programs and appear to share homologous signaling pathways and transcriptional regulatory mechanisms with higher vertebrates (Detrich et al., 1995; Thompson et al., 1998). Systematic, large-scale mutagenesis of the zebrafish, *Danio rerio*, provides a random approach to the discovery of novel hematopoietic genes (Donovan et al., 2000; Orkin and Zon, 1997; Parker et al., 1999; Paw and Zon, 2000), and the repertoire of developmental, genetic, and genomic technologies for functional analysis of newly discovered genes is large (Detrich et al., 1999a,b, 2004a,b). In this report, we introduce another fish system, the Antarctic Notothenioidei, whose unique taxa provide a compelling model for erythropoietic gene discovery. In particular, the hemoglobinless and erythrocyte-null icefishes (16 species, family Channichthyidae) (Cocca et al., 1995; Zhao et al., 1998), which diverged from erythrocyte-expressing notothenioids ∼5–8

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million years ago (Near, 2004; Near et al., 2003), provide a new resource for identifying erythropoietic genes via subtraction of their hematopoietic transcriptomes against those of their sanguineous relatives. Thus, our overall strategy is to use Antarctic fishes to isolate potential erythropoietic genes, to clone the zebrafish orthologs of these novel genes, and then to determine the functions of the genes in zebrafish embryos using reverse genetic technologies.

Here, we describe the structure and function of a novel RBCC/TRIM-B30.2 protein, Bloodthirsty (Bty), and demonstrate that it is required for erythropoiesis in the zebrafish. The *bloodthirsty* cDNA/gene (*bty*) was discovered as one of several B30.2-encoding cDNAs that were related to a subtraction product obtained by representational difference analysis (RDA) applied to the pronephric kidneys of two closely related Antarctic fishes, the erythrocyte-expressing Antarctic rockcod *Notothenia coriiceps* and the erythrocyte-null icefish *Chaenocephalus aceratus*. Both species expressed *bty* transcripts in their pronephric kidneys, the red-blooded rockcod at an ~10-fold greater steady-state level than that of the white-blooded icefish. *N. coriiceps* and *C. aceratus* Bty proteins contain 547 and 548 amino acid residues, respectively, and are ~90% identical. Bty is organized as sequential RING, B-Box 1, B-Box 2, coiled-coil, and B30.2 domains. To determine the function of *bty*/Bty in hematopoiesis, we cloned the orthologous *bty* cDNA from the zebrafish, *D. rerio*. Zebrafish *bty* encoded a 532-residue protein that was ~54% identical to the *N. coriiceps* and *C. aceratus* orthologs. Zebrafish *bty* transcripts were expressed by many tissues, including the intermediate cell mass, the site of primitive erythropoiesis, and the pronephric tubule, which develops into the adult kidney and is the site of definitive hematopoiesis. Knockdown of *bty* expression by injection of zebrafish embryos with antisense morpholino oligonucleotides (MOs) eliminated the production of erythrocytes of the primitive lineage, and this morphant phenotype was rescued by co-injection of synthetic *bty* mRNA containing an artificial 5′-UTR. *bty* knockdown reduced the abundance of several late-erythropoietic mRNAs, whereas expression of genes that mark early erythropoiesis, myelopoiesis, and vasculogenesis was unaffected by MO injection. These results suggest that *bloodthirsty* is required for terminal differentiation of erythroid progenitors of the primitive lineage.

Materials and methods

Fishes

Specimens of an Antarctic rockcod, *N. coriiceps*, and of the blackfin icefish, *C. aceratus*, were collected by bottom trawling from the *R/V Polar Duke* or from the *R/V Laurence M. Gould* near Brabant and Low Islands in the Palmer Archipelago. They were transported alive to Palmer Station, Antarctica, where they were maintained in seawater aquaria at −1.5 to +1°C. Tissues (pronephric kidney, spleen) were dissected and used immediately for preparation of RNA or tissue prints.

Wild-type zebrafish, *D. rerio*, were obtained from EKK Will Waterlife Resources (Gibsonton, FL) and were maintained in 40-l freshwater aquaria on a 14:10/light:dark photoperiod at 28–29°C (Westerfield, 2000). Embryos, obtained by mating two males with three or four females, were collected and placed in egg water (Westerfield, 2000) within the first hour post-fertilization (hpf). Some wild-type embryos (strain AB*) were obtained from the Zebrafish Facility of Children's Hospital, Boston. Staging of embryos followed the criteria of Kimmel et al. (1995).

Representational difference analysis (RDA)

Using pronephric-kidney cDNA amplicons from the erythrocyte-null icefish *C. aceratus* and the related, red-blooded notothenioid *N. coriiceps* as driver and tester, respectively, we applied cDNA RDA (Hubank and Schatz, 1994, 1999) to identify hematopoietic genes that were expressed differentially by the latter species. The complete protocol is described by Detrich and Yergeau (2004). As anticipated, *α*- and *β-globin* gene fragments were abundantly represented among the products (12 and 16.5%, respectively). Seventeen of the 45 contigs recovered were classified as unknowns ($E > 0$ for closest match). One of these, RDA 23, contained an open reading frame that encoded a portion of a novel B30.2 domain. The mRNA corresponding to the B30.2-encoding segment was strongly expressed by the pronephric kidney and spleen of *N. coriiceps*, the red-blooded fish, whereas it was almost undetectable in the same tissues of the white-blooded icefish, *C. aceratus* (data not shown). This B30.2-encoding fragment appears to be a bona fide example of the difference products that our protocol was designed to recover.

Cloning of the bloodthirsty (bty) gene from N. coriiceps, C. aceratus, and the zebrafish

Using a 307-bp B30.2 DNA probe derived from RDA 23 (Detrich and Yergeau, 2004), we screened an *N. coriiceps* spleen cDNA library (in λgt10) at moderate stringency for related cDNAs. Eight isolates were purified to the tertiary level, and their cDNA inserts were subcloned into the *Eco*RI site of the vector pBluescript KS (+). Phage and plasmid inserts were sequenced using the PRISM Ready Reaction Dye Deoxy Termination Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems 373A automated DNA sequencer (University of Maine DNA Sequencing Center). Two groups of B30.2-encoding cDNAs were recovered. The first ($n = 4$) corresponded to cDNAs whose inserts matched the sequence of the B30.2 probe exactly; the corresponding gene is under study and will be described elsewhere. The second serendipitous group ($n = 2$) encoded a B30.2 domain that was related, but not identical, to that of the

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