



Molecular and functional characterization of erythropoietin receptor of the goldfish (*Carassius auratus* L.)



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ABSTRACT

Erythropoietin receptor (EPOR) is a member of the class I cytokine receptor superfamily and signaling through this receptor is important for the proliferation, differentiation and survival of erythrocyte progenitor cells. This study reports on the molecular and functional characterization of goldfish EPOR. The identified goldfish EPOR sequence possesses the conserved EPOR ligand binding domain, the fibronectin domain, the class I cytokine receptor superfamily motif (WSXWS) as well as several intracellular signaling motifs characteristic of other vertebrate EPORs. The expression of *epor* mRNA in goldfish tissues, cell populations and cells treated with recombinant goldfish EPO (rgEPO) were evaluated by quantitative PCR revealing that goldfish *epor* mRNA is transcribed in both erythropoietic tissues (blood, kidney and spleen) and non-hematopoietic tissues (brain, heart and gill), as well as in immature erythrocytes. Recombinant goldfish EPOR (rgEPOR), consisting of its extracellular domain, dose-dependently inhibited proliferation of progenitor cells induced by rgEPO. *In vitro* binding studies indicated that rgEPO exists as monomer, dimer and/or trimer and that rgEPOR exists as monomer and/or homodimer, and when incubated together, formed a ligand–receptor complex. Our results demonstrate that goldfish EPO/EPOR signaling has been highly conserved throughout vertebrate evolution as a required mechanism for erythrocyte development.

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1. Introduction

Erythrocytes are unique cells that carry oxygen, a molecule absolutely required for energy production in all vertebrates. It follows that the maintenance of circulating erythrocyte volume is critical for sustaining the life of animals. The life span of mature human erythrocytes is estimated to be about 120 days (Shemin and Rittenberg, 1946); therefore, the continuous production of erythrocytes from hematopoietic stem cells must be tightly regulated. Erythropoiesis, the development of erythrocytes from their progenitor cells, is primarily controlled by erythrocyte growth factor, erythropoietin (EPO) binding to its receptor (EPOR) which causes proliferation, differentiation and survival of erythrocyte progenitor cells, including burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E) (reviewed by Constantinescu et al., 1999; Fisher, 2003).

EPOR is a member of class I cytokine receptor superfamily that includes the interleukin receptors, growth hormone receptors, and colony-stimulating factor receptors (Huising et al., 2006). The EPOR is composed of a signal peptide, a EPOR-ligand binding domain, a fibronectin type 3 domain, a class I cytokine receptor superfamily motif (WSXWS), a trans-membrane domain, and an intracellular cytoplasmic signaling domain containing two motifs termed Box 1 and Box 2, important for signal transduction (Constantinescu et al., 1999). EPO binding induces dimerization and reorientation of the cell surface EPORs, triggering activation of the Janus family protein tyrosine kinase 2 (JAK2) which transphosphorylates several intracellular EPOR tyrosine residues (Livnah et al., 1999; Remy et al., 1999), and leads to the activation of many signaling proteins, including signal transducer and activator of transcription factor 5 (STAT5), the PI-3 kinase and the protein tyrosine phosphatases SHP1 and SHP2 (Sasaki et al., 2000; Socolovsky et al., 1999; Uddin et al., 2000; Witthuhn et al., 1993; Richmond et al., 2005). These signaling pathways eventually lead to the proliferation, differentiation, survival, and maturation of erythrocyte progenitor cells. The expression of EPOR and signaling after EPO/EPOR interaction has been reported in non-erythroid tissues such as brain (Liu et al., 1997), retina (Grimm et al., 2002),

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heart (Wu et al., 1999), myoblasts (Ogilvie et al., 2000), and vascular endothelium (Anagnostou et al., 1994).

Recently, we identified and characterized goldfish EPO and found it to be functionally similar to its mammalian counterpart (Katakura et al., 2013). Recombinant goldfish EPO (rgEPO) promoted the proliferation, differentiation and survival of erythrocyte progenitor cells and up-regulated *epor* mRNA levels in progenitor cells. Studies using zebrafish also have reported the conservation of EPO/EPOR and STAT5 signal transduction in erythropoiesis (Paffett-Lugassy et al., 2007). Moreover, EPO and EPOR of *Xenopus laevis* were also shown to be involved in erythropoiesis (Aizawa et al., 2005; Nogawa-Kosaka et al., 2010). Interestingly, the EPO/EPOR system appears to be well conserved throughout the evolution of vertebrates, despite the fact that lower vertebrates such as fish, amphibians, reptiles and birds, have nucleated erythrocytes while mammals have enucleated erythrocytes.

In this study, we cloned and functionally characterized goldfish EPOR. Goldfish *epor* mRNA expression was measured in goldfish tissues, different cell subpopulations and in FACS-sorted kidney progenitor cells. We also measured the expression of *epor* in cells from the primary kidney macrophage (PKM) cultures treated with rgEPO, to determine whether rgEPO signaling maintained erythroid lineage cells *in vitro*. Further, we examined whether the addition of soluble recombinant goldfish EPOR (the extracellular domain of EPOR) abrogated the rgEPO-induced proliferation of the primary kidney progenitor cells.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus* L.) were obtained from Aquatic Imports (Calgary, AB). Fish were maintained in tanks with a continuous flow water system at 20 °C and with a 14 h light/10 h dark period in the aquatic facilities of Biological Sciences building at the University of Alberta. Fish were fed daily and were acclimated for at least 3 weeks prior to use in the experiments. Prior to handling, fish were sedated using a tricaine methane sulfonate (TMS, syn MS-222) solution of 40–50 mg/L in water. The animals in the Aquatic Facility were maintained according to the guidelines of the Canadian Council of Animal Care (CCAC).

2.2. Isolation and establishment of goldfish primary kidney macrophage (PKM) cultures

Goldfish (10–15 cm) were anesthetized with TMS, and killed. The isolation and cultivation of goldfish kidney leukocytes in complete NMGL-15 medium containing 5% carp serum and 10% newborn calf serum was performed as previously described (Neumann et al., 1998, 2000). Cells were cultured at 20 °C. The primary kidney macrophage (PKM) cultures consisted of heterogeneous populations of cells including early progenitors (R1 gated cells), monocytes (R3 gated cells) and mature macrophages (R2 gated cells) as determined by flow cytometry, morphology, cytochemistry and function (Neumann et al., 1998, 2000).

2.3. DNA sequencing and in silico analyses of goldfish *epor*

The initial partial sequence for goldfish *epor* was identified using homology-based primers (Integrated DNA Technologies, IDT) designed against corresponding carp sequences in the NCBI database (AB671215). RACE PCR (BD Sciences, Clontech) was performed to obtain a full open reading frame for *epor*. All amplicons were gel purified using the QIA Gel Extraction kit (Qiagen) and cloned into the TOPO TA pCR2.1 vector (Invitrogen). Colony PCR

was used to identify positive colonies using the vector specific M13 forward and reverse primers, plasmids isolated using the QIA-spin Miniprep kit (Qiagen) and sequenced using a BigDye terminator v3.1 cycle sequencing dye and a PE Applied Biosystems 377 automated sequencer. Single pass sequences were analyzed using 4peaks software (<http://mekentosj.com/4peaks/>) and sequences aligned and analyzed using BLAST programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The complete list of primers used for homology-based PCR, RACE PCR, Q-PCR, sequencing and recombinant protein expression is shown in Supplemental Table 1.

EPOR protein sequences from fish, amphibian and mammals were aligned using Clustal Omega software (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Signal peptide regions of respective EPOR proteins were identified using the SignalP 4.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) and conserved motifs were predicted using the SMART server (<http://smart.embl-heidelberg.de/>). Phylogenetic analysis was conducted using Clustal X and NJ-plot software using the neighbor joining method and bootstrapped 10,000 times, with values expressed as percentages Supplemental Fig. 1). The full-length sequence of goldfish EPOR has been submitted to GenBank (KC595243).

2.4. Isolation of goldfish splenocytes, peripheral blood leukocytes and kidney neutrophils

Splenocytes from individual goldfish ($n = 4$) were obtained by gently homogenizing the spleen through a wire mesh screen using NMGL-15 medium containing heparin and penicillin/streptomycin (Pen/Strep). Cell suspensions were layered over 51% percoll and centrifuged at 430×g for 25 min. Cells at the interface were collected and any residual red blood cells were lysed using red blood cell lysis buffer (144 mM NH₄Cl, 17 mM Tris, pH 7.2). Remaining cells were washed twice with incomplete NMGL-15 medium.

To isolate peripheral blood leukocytes, four goldfish were bled from the caudal vein using a 25G needle and heparinized syringe to prevent clotting. The blood was transferred into a capillary glass tubes. After sealing one side with clay, the tubes were centrifuged at 1500×g for 5 min. Leukocytes were collected by cutting the tubes 2 mm below the leukocyte layer and suspended in NMGL-15 medium. After centrifugation at 250×g for 10 min to pellet the leukocytes, the supernatant was discarded and residual red blood cells were lysed using the red blood cell lysis buffer. Cells were washed twice with incomplete NMGL-15 to remove residual red blood cell lysis buffer.

Kidney neutrophils were isolated and cultured overnight in complete NMGL-15 medium containing 5% carp serum and 10% newborn calf serum at 20 °C, as described (Katzenback and Belos-ovic, 2009). Following overnight incubation to remove residual contaminating adherent monocytes/macrophages, suspension of cells containing neutrophils was collected and washed twice with NMGL-15.

2.5. Sorting of goldfish R1 progenitor cells

Freshly isolated leukocytes from goldfish kidney were re-suspended to a concentration of 5–10 × 10⁶ cells/mL in complete NMGL-15 for cell sorting on a FACS Aria flow cytometer (Becton Dickinson) in the Department of Medical Microbiology and Immunology, University of Alberta, Flow Cytometry Facility. R1 gated cells, consisting of mainly heterogeneous early progenitors, were sorted based on their small size and low internal complexity into 15 mL tubes containing 7 mL of complete NMGL-15 (supplemented with serum) medium containing 100 U/mL of penicillin/100 µg/mL of streptomycin and 100 µg/mL of gentamicin and then washed twice with incomplete NMGL-15.

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