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# Lipopolysaccharide primes the respiratory burst of Atlantic salmon SHK-1 cells through protein kinase C-mediated phosphorylation of p47phox

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ABSTRACT

The superoxide-producing NADPH oxidase complex of phagocytes plays a crucial role in host defenses against microbial infection. NADPH oxidase consists of a membrane heterodimeric protein, composed of gp91phox and p22phox, and the cytosolic proteins, p40phox, p47phox and p67phox. In the present study, we clone and sequence the full-length cDNAs coding for the Atlantic salmon (*Salmo salar*) phagocyte NADPH oxidase components, p47phox, p67phox and gp91phox, using a homology cloning approach. The sequences of these cDNAs showed that the *S. salar* p47phox, p67phox and gp91phox genes contained single open reading frames, which encoded predicted proteins of 413, 504 and 565 amino acids, respectively. Comparison of the deduced amino acid sequences showed that the *S. salar* p47phox, p67phox and gp91phox sequences shared 51, 45 and 68% identity with those of human components, respectively. Despite this relatively low homology between salmon and mammalian NADPH oxidase subunits, their functional domains are highly conserved. We also found that the mRNA levels of p47phox, p67phox and gp91phox expression were higher in immune-related tissues, such as kidney, spleen and gill. In addition, infection of the salmon macrophage cell line SHK-1 with *Piscirickettsia salmonis* induced the expression of p47phox, but had no effect on p67phox and gp91phox expression. Finally, we show for the first time in fish that activation of macrophages with lipopolysaccharide promotes the activation of protein kinase C, which in turn phosphorylates p47phox, leading to NADPH oxidase activation and reactive oxygen species generation. Collectively, these results suggest that the mechanisms of activation of phagocyte NADPH oxidase are well conserved from fish to mammals.



## 1. Introduction

Reactive oxygen species (ROS) generated by NADPH oxidase (Nox) are not only involved in bacterial killing by phagocytes, but also play key roles in signaling and host defense in a variety of cell types. In mammals, the pivotal role of NADPH oxidase in microbial killing is highlighted by the fact that neutrophil killing is compromised under anaerobic conditions (Mandell, 1974) and because its dysfunction causes chronic granulomatous disease (CGD), which is characterized by a profound predisposition to bacterial and fungal infections (Thrasher et al., 1994; Winkelstein et al., 2000).

Leukocyte NADPH oxidase catalyzes the reduction of oxygen to superoxide anion ($O_2^-$) at the expense of NADPH in phagocytes and B lymphocytes (Chanock et al., 1994). Dismutation of $O_2^-$ by superoxide dismutases generates hydrogen peroxide, which is a source of hydroxyl radicals (Robinson and Badwey, 1998). In mammals, the best studied oxidase is the phagocyte NADPH oxidase (Phox), a multicomponent enzymatic complex responsible for the production of large amounts of superoxide anions during oxidative and/or pathological conditions, both in cultured cells and in infected organisms (Chanock et al., 1994). Activation of the NADPH oxidase complex requires the coordinated recruitment of several cytosolic subunits to the phagocytic membrane and their assembly with the membrane-bound cytochrome $b_{558}$ (Bokoch, 1994). Cytochrome $b_{558}$ is a flavohemoprotein comprising two membrane-associated NADPH oxidase components, gp91phox and p22phox (Huang et al., 1995). Three of the cytosolic NADPH oxidase components, p47phox, p67phox and a second low molecular weight GTP-binding protein, Rac2, have been shown to translocate from the cytosol to the membrane during NADPH oxidase assembly. Additionally, a cytosolic protein known as p40phox has been shown to associate with p47phox and p67phox, a pair of proteins that stabilize the 240-kDa complex of cytosolic oxidase in resting cells (Vignais, 2002).

The innate immune system is the only defense system of invertebrates and a fundamental defense mechanism of fish. Nevertheless, there have been very few studies on one of the main phagocyte

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killing effector mechanisms, namely the NADPH oxidase complex, in teleosts. The studies that exist were mainly focused on the molecular cloning of the genes coding for some NADPH oxidase components and their expression; see, for example, the study of Mayumi et al. (2008) in carp (*Cyprinus carpio*) and Inoue et al. (2004) in the Japanese pufferfish (*Takifugu rubripes*). In addition, some recent studies have dealt with the phylogenetic analysis of the Nox/Duox family of oxidases in several model species (Kawahara et al., 2007; Kawahara and Lambeth, 2007), such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and pufferfish (*Tetraodon nigroviridis*).

In the present study, we describe the cloning, sequencing, expression and phylogenetic analysis of the cDNAs coding for p47phox, p67phox and gp91phox in Atlantic salmon. In addition, we demonstrate for the first time in fish using a combination of *in vitro* and *in vivo* assays that stimulation of macrophages with lipopolysaccharide (LPS) resulted in the activation of protein kinase C (PKC), which, in turn, phosphorylated p47phox, leading to the activation of NADPH oxidase and the generation of ROS.

## 2. Materials and methods

### 2.1. Animals

Specimens (30 g mean weight) of Atlantic salmon (*S. salar*) were obtained from Novartis Puerto Varas, Chile. The fish were certified to be free of infectious salmon anemia virus and *P. salmonis*. Tissue samples were directly collected in the fish farm and immersed in TRIzol reagent (Invitrogen) for further analysis.

### 2.2. Cell culture

SHK-1 is a cell line from *S. salar* head kidney, which exhibits macrophage properties (Dannevig et al., 1997). The cells were cultured at 18 °C in 75 $cm^2$ tissue-culture-treated flasks (Costar), in L-15 medium (500 ml with 300 mg/l L-glutamine) supplemented with 500 μl gentamicin sulfate (50 mg/ml in distilled water), 365 μl 2-mercaptoethanol (55 mM in Dulbeco's phosphate buffered saline) and 5% fetal bovine serum (FBS). All media components were purchased from Gibco. Confluent flasks were subcultured weekly by dividing cells and medium evenly between two flasks and adding an equal volume of new media to each flask. The cells used in this study were between 58 and 60 passages.

### 2.3. Growth of P. salmonis in SHK-1 cells

*P. salmonis* isolate LF-79 was obtained from an outbreak of salmonid rickettsia septicemia (SRS) in Atlantic salmon in Puerto Montt (Chile) in September 2009 and was cultured in CHSE-214 cells (ECACC 91041114) in Eagle's minimal essential medium (MEM) with 4 mM glutamine and 10% FBS in the absence of antibiotics.

The concentration of infectious units (IU) of *P. salmonis* in cell cultures was determined by plaque assay (Cvitanich et al., 1991), in which serial dilutions of homogenates of tissue-culture cells in Eagle's MEM were added to duplicate wells of monolayer cultures of CHSE-214 cells in Costar 24-well culture plates. After culture for 10 days at 18 °C, the medium was removed, cells were Giemsa stained, and plaques resulting from *P. salmonis* replication were counted. Each plaque was considered to represent 1 IU of *P. salmonis* in the original culture fluid.

SHK-1 cells were infected with $10^4$ IU/ml of *P. salmonis* for 1, 3 and 7 days. The cytopathic effect (CPE) and PCR assay for *P. salmonis* 16S rRNA (5′-a g g g a g a c t g c c g g t g a t a-3′ and 5′-a c t a c g a g g c g c t t t c t c a-3′ (Karatas et al., 2008)) demonstrated the presence and activity of the bacteria in SHK-1 cells.

**Table 1**
Primer used in PCR for amplification of Atlantic salmon NADPH subunits.

| Target | Name | Sequence 5′–3′ |
|---|---|---|
| p47phox | p47-Fl | ATGGAGGAAATCTATGTCAGGC |
| | p47-Rl | TGATATAGAGTTCTCCTGCATAGTTGG |
| | p47-F2 | ATATTGGAGACTTGGTGGAGATTG |
| | p47-R2 | TCAGTTCGTCTTGCGGAC |
| p67phox | p67-Fl | ATGTCATTTGTGAATACTCTAAAACAG |
| | p67-Rl | GGACAAACTCAAAGAGGACAGTG |
| | p67-F2 | CTGTCCTCTTTGAGTTTGTCCC |
| | p67-R2 | TTATTTATCTTTCATGGAAACTTCTTC |
| gp91phox | gp91-Fl | ATGGGCAACTTTGCTGCC |
| | gp91-Rl | GGATGATGTAATGATGAGGATGAG |
| | gp91-F2 | TTAACGGGCGTGGTCATC |
| | gp91-R2 | CAGTTTCCATGCCTCCTGG |
| | gp91-F3 | CAAGAACGAGACCCAGGAG |
| | gp91-R3 | TCAGAAGTTTTCCTTGTTGAAGATG |

### 2.4. Cell stimulation

SHK-1 cells were seeded at approximately $4.0 \times 10^6$ cells/flask in L-15 medium supplemented as described above. Cells were stimulated as described previously (Brubacher et al., 2000; Zou et al., 2000). Briefly, following a 48-h period after subculturing to minimize manipulation-induced gene expression, 10 ml of fresh medium with or without 1 μg/ml LPS was added to each flask. In some experiments, cells were pretreated for 1 h with 10 μM calphostin A (PKC inhibitor) or 10 μM apocynin (NADPH oxidase inhibitor) (both from Sigma–Aldrich). Treatments were carried out in triplicate and cells were stimulated for 4 h prior to evaluate the respiratory burst activity. None of the treatments used in this study affected cell viability, morphology or adherence properties, as determined by trypan-blue exclusion and light microscopy. In all the experiments, cell viability was >95% at the end of the incubation period.

### 2.5. cDNA cloning

Total RNA was extracted from tissue and SHK-1 cells using TRIzol reagent following the manufacturer's instructions and treated with DNase I, amplification grade (1 U/μg RNA; Invitrogen). The SuperScript III RNase H-reverse transcriptase (Invitrogen) was used to synthesize the first strand of cDNA with an oligo$(dT)_{18}$ primer from 1 μg of total RNA at 50 °C for 50 min. To obtain a partial sequence of the cDNA coding for the *S. salar* NADPH oxidase subunits, primers for each subunits were designed based on EST sequences of *S. salar* (Atlantic salmon) and conserved regions in rainbow trout (*Oncorhynchus mykiss*) (Table 1). PCR reactions were performed under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 60 s. The PCR products were sequenced with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). To obtain the entire cDNA sequence, both strands of each PCR product were sequenced according to the manufacturer's instructions.

### 2.6. Sequence analysis

Multiple alignments of NADPH oxidase components were obtained with GENETYXVersion 6.1 (GENETYX). Unrooted phylogenetic trees were constructed based on the amino acid sequence alignments (http://www.genebee.msu.su/services/phtree_reduced.html) (Yushmanov and Chumakov, 1988). Potential motifs and domains were searched using the SMART database