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Peritoneal cavity phagocytes from the teleost sea bass express a glucocorticoid receptor (cloned and sequenced) involved in genomic modulation of the *in vitro* chemiluminescence response to zymosan ☆

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Abstract

To gain further insight into the role of cortisol in fish innate immune responses, we cloned and sequenced a 2592 bp cDNA from sea bass (*Dicentrarchus labrax*) peritoneal leukocytes (PCLs) encoding a glucocorticoid receptor (DlGR1). The deduced aminoacid sequence displayed that DlGR1 belong to a multigenic family of steroid hormone receptors, and exhibited high homology (80%) to the Burton's mouth breeder (*Haplochromis burtoni*) HbGR1. The DlGR1 functional domains presented homologies with those of several vertebrate species. In *situ* hybridization assay revealed that DlGR1 was expressed in macrophages and neutrophils from the peritoneal cavity. Since in a previous paper, sea bass PCL chemiluminescence response (CL) has been related to increased respiratory burst of phagocytes stimulated with zymosan, PCLs, pre-incubated *in vitro* with cortisol at various concentrations, were assayed for their CL response. Dose-dependent cortisol inhibitory effects, and significant competitive activity of a low concentration of mifepristone (RU486), a glucocorticoid-receptor blocker, supported that cortisol–GR interaction was involved in modulating CL response *via* a genomic pathway. Results also indicated that cortisol could be effective through an additional not-genomic way, and showed that high doses of RU486 exerted an inhibitory effect on PCL chemiluminescence activity.

Keywords: *Dicentrarchus labrax*; Peritoneal cavity leukocytes; Phagocytes; Hydrocortisone; RU486; Glucocorticoid receptor; DlGR1; GR cDNA sequence; GR mRNA expression

1. Introduction

In vertebrates, corticosteroid hormones (mineralcorticoids MCs, and glucocorticoids GCs) are essential for normal development and maintenance of basal and stress-related homeostasis. They regulate a broad range of metabolic processes and physiological functions through two classes of corticosteroid receptors (MR and GR). GCs exert a part of their effects genomically *via* passive diffusion through the cell membrane, occupation of receptors located in the cytosol, translocation of the GC-receptor complex into the nucleus, combination with GCs specific responsive elements present in gene promoters, or by interacting with NF-κB transcription factor (McKay and Cidlowski, 1999) inducing or repressing transcription which leads to biological function (Cupps and Fauci, 1982).

GCs influence the cells of innate immunity such as monocytes/macrophages, neutrophils and NK cells (Schmidt et al., 1999), down-regulate monocyte-secreted proinflammatory mediators (Joyce et al., 1997; Breuninger et al., 1993) and inhibit adherence, chemotaxis, phagocytosis and cytotoxic activity (McGillen and Phair, 1979; Roth and Kaeberle, 1981; McEwen et al., 1997; Rogers et al., 1999). In addition, after *in vivo* or *in vitro* exposures to GCs, bactericidal and fungicidal ability (Rinehart et al., 1975), cytokine production, MHC class II expression (Snyder and Unanue, 1982) and tumoricidal activity (Hogan and Vogel, 1988; Keil et al., 1995) of

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humans and murine macrophages were decreased. *In vivo*, high levels of plasma cortisol produced by stressors can lead to divergent responses including a strong immunosuppressive effect (Cupps and Fauci, 1982; Rinehart et al., 1975). In fish, cortisol (hydrocortisone) is the major corticosteroid with both GC and MC activities. *In vivo* and *in vitro* experiments have shown immunosuppressive property of cortisol in reducing the normal ability of striped bass phagocytes to generate a respiratory burst response, assayed as chemiluminescence (CL) reaction when exposed to bacteria or phorbolester (Stave and Roberson, 1985), in modulating apoptosis and proliferation of carp B lymphocytes (Verburg-Van Kemenade et al., 1999), in putting down mitogen-induced proliferation of rainbow trout lymphocytes (Tripp et al., 1987), and in suppressing tilapia leukocyte phagocytosis (Law et al., 2001).

GRs have been cloned from human (Hollenberg et al., 1985), mouse (Danielsen et al., 1986), rat (Miesfeld et al., 1986) and *Xenopus* (Gao et al., 1994a), and are considered members of a large receptor family that include androgen, progesterone, estrogen, and retinoid receptors. Sequences of GR cDNAs contain four functional domains, respectively, responsible for transcriptional activation, DNA-binding, nuclear localization, and hormone-binding (Giguere et al., 1986; Oro et al., 1988). In fish, evidence mainly came from salmonids by various approaches, and GRs have been identified into the gills (McLeese et al., 1994; Shrimpton and Randall, 1994; Shrimpton et al., 1995), liver (Vijayan et al., 1993; Pottinger et al., 1994), brain (Lee et al., 1992; Knoebl et al., 1996), and intestine (DiBattista et al., 1984). In rainbow trout (*Oncorhynchus mykiss*), the translated product of a cDNA, with a high sequence identity to mammalian GRs, exhibited cortisol-binding activity *in vitro* (Ducouret et al., 1995). Greenwood et al. (2003) sequenced two distinct GR cDNA (HbGR1 and HbGR2) from tissues of Burton's mouth breeder (*Haplochromis burtoni*). In addition, transcripts were found in a number of tissues (Takeo et al., 1996; Teitsma et al., 1997; Uchida et al., 1998; Perry, 1997), whereas data are lacking on GR expression in fish leukocytes.

According to Scott and Klesius (1981) and Stave et al. (1983), during phagocytosis the respiratory burst, due to an increased oxygen consumption, can be measured using the phagocyte chemiluminescence amplified with luminol (CL). In a previous paper, we related *in vivo* increased sea bass (*Dicentrarchus labrax*) plasma cortisol levels (700–1000 ng/ml), due to stress caused by confinement and high density, to the depression of spontaneous cytotoxic activity of peritoneal eosinophilic granulocytes (EGLs), whereas CL of peritoneal cavity leukocytes (PCLs) appeared weakly decreased in confined fish at a non significant level (Vazzana et al., 2002). However, when all the examined variables (CL and cytotoxic activity of PCLs, plasma glucose, cortisol levels and osmolarity) were evaluated by statistical discriminant analysis, CL values contributed in discriminating stressed fish group. Vazzana et al. (2003) showed that CL of sea bass PCLs could be related to an enhanced respiratory burst of phagocytes stimulated with β-glucan.

In the present study, we show that a brief *in vitro* cortisol treatment suppressed, in a dose-related fashion, the CL response of sea bass PCLs, due to β-glucan-stimulation. Competitive activity of a low concentration of mifepristone (RU486), a glucocorticoid-receptor blocker, gave evidence that cortisol–GR interaction was involved in modulation of the CL. Results also indicated that cortisol could be effective through a not-genomic way, and showed that high doses of RU486 exerted a suppressive effect on PCL chemiluminecence activity. A GR cDNA from PCLs was isolated and sequenced (DlGR1) supporting the presence of a genomic cortisol modulation pathway. The deduced aminoacid sequence appeared to be homologue to the known fish GR cDNAs and exhibited high homology (80%) to the Burton's mouth breeder (*H. burtoni*) HbGR1. DlGR1 presumptive functional domains displayed that both DNA and hormone-binding domains contained highly conserved sequences. In the resulting phylogenetic tree, DlGR1 appeared to be included into the multigenic family of corticosteroid hormone receptor. Finally, *in situ* hybridization assay displayed that peritoneal cavity macrophages and neutrophils expressed DlGR1.

2. Materials and methods

2.1. Animals, and cell suspension preparations

Sea bass (200–250 g) were obtained from a commercial fish farm (Petrosino, TP, Italy). Fish were anaesthetized with 0.05% 3-aminobenzoic acid ethyl ester (Sigma–Aldrich, Germany) in seawater, and sampled at the farm. The culture medium contained: Leibovitz L-15 medium, 2% foetal calf serum, 100 U penicillin ml^{-1}, 100 U streptomycin ml^{-1}, and 10 U heparin ml^{-1}. Culture medium components were from Gibco Life Technologies.

PCLs were obtained as follows: after disinfection of the fish ventral surface with 70% ethyl alcohol, 15 ml of isotonic (370 mOsm kg^{-1}) Leibovitz medium were injected into the peritoneal cavity. After massaging for 10 min, the medium containing the PCLs was harvested with a syringe, and PCLs were separated centrifuging at 400*g* for 10 min at 4 °C. Since no inflammatory agents were contained in the injected medium, the peritoneal cells only received stimulation by the injection procedure. Osmolarity of serum and medium were measured by an osmometer (Röebling).

Sea bass PCLs were previously identified (Vazzana et al., 2003) and classified according to Rowley (1990). PCL preparations approximately contained: 26% macrophages characterized by prominent vacuoles and a typical irregular outline when smeared on the slide, 32% lymphocytes, 22% neutrophils (identified by the peroxidase reaction), and 18% eosinophil granulocytes. Macrophages and neutrophils phagocytosized yeast.

According to Vazzana et al. (2003), enriched leukocyte populations were contained in the bands separated through a continous 55% Percoll density gradient centrifugation of PCL preparations. Macrophages and neutrophils were enriched in Band 1 (about 34% and 30%, respectively), and eosinophil granulocytes in Band 2 (85–98%).

2.2. Chemiluminescence (CL) assay

Activation of sea bass phagocytes was measured as enhanced ROI production using a luminol-amplified CL assay as previously reported (Vazzana et al., 2003). To detect CL, a luminol working solution was prepared (0.014 g luminol, 0.78 g potassium hydroxide, and 0.618 g boric acid added to 10 ml of distilled water), diluted 1:100 in HBSS (Hanks balanced salt

solution: NaCl 190 mM, KCl 5.36 mM, glucose 5.54 mM, KH_2PO_4 0.44 mM, Na_2HPO_4 0.56 mM; pH 7.6, 370 mOsm kg^{-1}) and stored in the dark. Zymosan, a β-glucan of yeast wall, was prepared as reported in Scott and Klesius (1981), by boiling for 30 min in phosphate-buffered saline (PBS), followed by centrifugation for 5 min at 400*g*. The pellet was suspended in 50 ml of PBS, to a final concentration of 1 mg ml^{-1}, and stored until use, with a maximum storage time of 2 weeks. The cell number was standardised to 2.5×10^5 cells ml^{-1} in HBSS. Aliquots of 0.5 ml leukocyte suspension were mixed in propylene vials with 0.5 ml luminol working solution. The reaction was initiated by the addition of 0.5 ml zymosan preparation. CL was estimated in a liquid scintillation counter (LS 1800, Beckman) and CPM values recorded. Each sample was run in triplicate. Controls were performed as samples of reaction mixture in which luminol working solution, or zymosan preparation, was replaced by culture medium.

2.3. In vitro PCLs treatments with hydrocortisone (HC) and/or mifepristone (RU)

Stock solutions of HC or RU were prepared by dissolving them in absolute ethanol to 2.7×10^{-3} and 1×10^{-3} M, respectively. PCLs from ten fish, in separate experiments, were incubated into the medium containing HC (Sigma–Aldrich, Germany) at different final concentrations (2.7×10^{-7}, 2.7×10^{-6}, and 2.7×10^{-5} M). The 2.7×10^{-6} M HC concentration was in accordance with cortisol maximum level found in the plasma of stressed sea bass (Vazzana et al., 2002). The cultures were incubated at 18 °C in 5% CO_2 for 1 h and, soon afterwards, 0.5 ml of a zymosan preparation and luminol working solution were added. A short treatment (1 h) avoided unwanted effects on the cells. Mifepristone [11β-(4-dimethylamino) phenyl-17β-hydroxy-17-(1-propynyl) estra-4, 9-dien-3-one; RU486] a steroid analogue with high affinity for GR, was tested for its competitive activity towards cortisol (Van Voorhis et al., 1989; Long et al., 2005). PCLs were incubated (1 h) with different concentrations (10^{-5}, 10^{-8}, and 10^{-11} M) of RU prior to the HC treatment.

Controls without cortisol or RU were performed by preparing PCLs suspension in a reaction medium containing 0.1% ethanol, and maintained in the same experimental conditions of the treated samples.

Cell viability was estimated by trypan blue exclusion test (0.01%). In all the assays, percent of dead cells were less than 5%.

Chemicals were from Sigma–Aldrich.

2.4. Isolation of GR cDNA

Total RNA was isolated from PCLs by using a RNAqueous™-Midi Kit purification system (Ambion) and reverse transcribed by the Kit Ready to Go T-primed first-strand using random primers (Amersham-Pharmacia Biotech). Amplification was performed according to Greenwood et al. (2003), using 10 μM of the degenerate primers: 5′-AGT GCT CCT GGC TGT TYC TNA TG-3′ (RT1) and 5′-TTT CGG TAA TTG GTT GCT GAT GAT-3′ (RT2) designed on highly conserved vertebrate GR sequences that characterized the hormone-binding domain. A single band of 468 bp in size was detected. This product was cloned into the pCR™IIvector (TA cloning Kit, Invitrogen) and its sequence revealed 90% homology with the *H. burtoni* HbGR1 nucleotide sequence reported by Greenwood et al. (2003), and 73% homology with human GR. Therefore the 5′ and 3′ ends of cDNA were cloned by using GeneRacer Kit (Invitrogen) in accordance with the manifacturer instructions. To increase specificity and reduce background amplification, the first round of PCR, with RT1 primer for 3′ RACE and RT2 for 5′ RACE, was performed with a touchdown protocol. The second round of PCR (nested PCR) was carried out with 1 μl of the product obtained from the first PCR as template. Specific nested primers designed according to the sequence of the above reported 468 bp band (10 μM D1 5′-CGC GCT TCA CGA TGG CTT TCC CC-3′, 10 μ M S1 5′-GCA ACG GCA ACA TGC TCT GCT TCG C-3′) were used, and 30 cycles at the annealing temperature of 65 °C were carried out. Finally, PCR products were cloned into the pCR™IIvector (TA cloning Kit, Invitrogen) and sequenced.

2.5. GR splice variant analysis

To determine whether DLGR1 was expressed as a splice variant, we designed the following primers spanning the DNA-binding domain: upper primer, 5′-AGGACAGGGCTACCGATTTG-3′; lower primer, 5′-GTTCTCTCGTCACTTTCCCG-3′. An amplified product of 365 bp was obtained using the following conditions: 30 cycles at the annealing temperature of 60 °C.

2.6. cDNA sequencing and sequence analysis

To determine the nucleotide sequence, suitable amounts of lyophilised samples were analysed by CRIBI (Biotechnology Centre of the University of Padua, Italy, http://bmr.cribi.unipd.it, ABI PRISM-DNA sequencer, Applied Biosystems). To examine DNA and deduced amino acid sequence homologies, multiple alignments were accomplished with the Clustal W program (Thompson et al., 1994). The alignment of DlGR1, HbGr1 and RtGR1 and RtGR2 of *Oncorhynchus mykiss*, *Xenopus laevis* and human GR was produced with ClustalX v.1.81 (Thompson et al., 1997) and similarity shaded with GeneDoc v.2.6.002 (Nicholas and Nicholas, 1997).

A phylogenetic tree was constructed on steroid receptor amino acidic sequences, by the Neighbor-Joining method (NJ), and 1000 bootstrap replicates were performed.

2.7. In situ hybridization assay

Digoxigenin-11-UTP-labeled riboprobes (DIG-riboprobe) were developed according to the instructions of the manufacturer (Roche Diagnostic, Mannheim, Germany), and used at a final concentration of 1 μg/ml (100 ng probe/slide). The riboprobe included the transcriptional activation domain GR cDNA (1.0–1300 nucleotide sequence). PCLs (2×10^6/ml) were layered on a slide, and fixed in Bouin solution (saturated picric acid:formaldehyde solution:acetic acid = 15:5:1) according to Le Guellec (1998). After washing in PBST (PBS containing 0.1% Tween 20), the hybridization was carried out overnight with hybridization buffer (50% formamide, 5× standard saline citrate (SSC) buffer from Sigma–Aldrich, 50 μg/ml heparin, 500 μg/ml yeast tRNA, and 0.1% Tween 20). Incubation temperature was at 42 °C. After washings with PBST followed by 0.3% SSC/1% Tween 20 (twice for 10 min each), the anti-DIG Fab-AP (Roche Diagnostic) 1:500 was added, and, after 1h incubation at r.t., washed with PBST. Finally, the PCLs were incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma–Aldrich). Control experiments were run using the corresponding sense cRNAs.

After *in situ* hybridization assay, some PCL smears were treated with Giemsa stain for 10 min, and observed under a light microscope Leica DMRE.

2.8. Statistical methods

Results were expressed as a mean percentage of the control value ± SD. To determine the statistical probability the obtained data were examined by the Mann–Whitney *U* test (Dinneen and Blakesley, 1973), $P < 0.05$ being the lowest significance level. Values came from ten separated experiments, each of them performed in triplicate.

3. Results

3.1. Hydrocortisone affects CL response of zymosan stimulated PCLs

PCLs were sensitive to zymosan activation as showed by an enhanced CL (from $198 \times 10^3 \pm 30$ to $710 \times 10^3 \pm 77$ CPM). To verify that HC modulate the CL, *in vitro* assays were undertaken incubating 2.5×10^5 cells/ml with several final concentrations of HC. As shown in Fig. 1, after 1 h

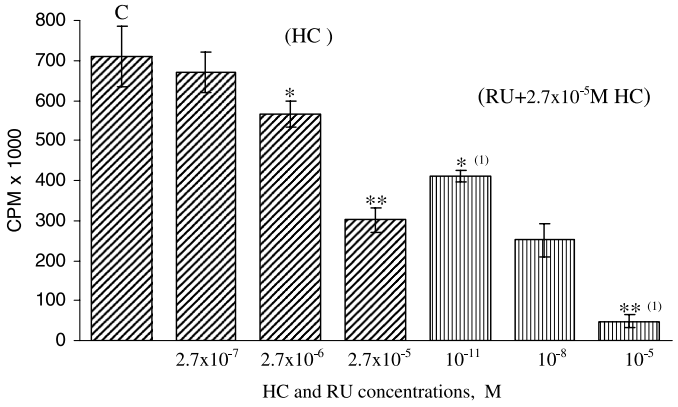

Fig. 1. Chemiluminescence response (CPM) of PCLs. (HC): leucocytes (2.5×10^5/ml) from sea bass peritoneal cavity following 1 h *in vitro* treatment with various hydrocortisone (HC) concentrations, ▨ HC: 2.7×10^{-7}, 2.7×10^{-6}, and 2.7×10^{-5} M. Control (C) was performed by preparing a leukocyte suspension in a medium containing 0.1% ethanol, without hydrocortisone. (RU+HC): After 1 h mifepristone (RU) treatment, PCLs were treated (1 h) with HC (HC+RU) at different concentrations ▥ (10^{-11}, 10^{-8}, and 10^{-5} M RU plus 2.7×10^{-5} M HC). Phagocyte CL response was estimated, after activation with zymosan, in a liquid scintillation counter (LS 1800, Beckman) and CPM values recorded. Cell mortality was lower than 5%. Values are averages of ten separated experiments. $^{*}P < 0.05$; $^{**}P < 0.01$. (1): compared to CL response of 2.7×10^{-5} M HC treated samples.

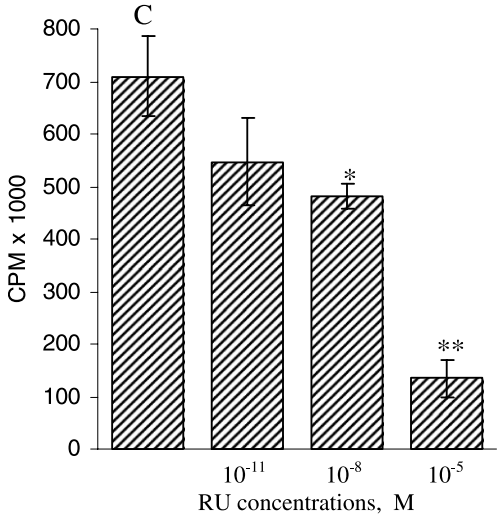

Fig. 2. Chemiluminescence response (CPM) of leucocytes from sea bass peritoneal cavity after *in vitro* treatment (1 h) with various mifepristone (RU) concentrations (10^{-11}, 10^{-8}, and 10^{-5} M). Phagocyte CL response was estimated, after activation with zymosan, in a liquid scintillation counter (LS 1800, Beckman) and CPM values recorded. Control (C) was performed by preparing a leukocyte suspension in a medium containing 0.1% ethanol, without RU486. Cell mortality was lower than 5%. Values are averages of ten separated experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

incubation, 2.7×10^{-6} or 2.7×10^{-5} M HC caused a dose-related significant decrease ($P < 0.05$ and $P < 0.01$, respectively) of the CL response as compared to the control (PCLs treated with zymosan alone). A lower HC concentration (2.7×10^{-7} M) did not significantly diminish the CL response.

3.2. Effect of glucorticoid-receptor antagonist

To verify that the HC action was GR-dependent, mifepristone was used as an antagonist. As shown in Fig. 1, after incubation with 10^{-11} M RU (final concentration), PCLs became significantly ($P < 0.05$) less sensitive to the subsequent HC (10^{-5} M) treatment. When assayed in the absence of HC, 10^{-11} M, RU exerted a weak not significant depressive effect on the CL activity, whereas higher concentrations (10^{-8}, 10^{-5} M) significantly ($P < 0.05$ and $P < 0.01$, respectively) reduced the CL values in a dose-dependent fashion (Fig. 2) reaching levels lower ($P < 0.01$) than those observed after treatment with the highest HC dose (10^{-5} M, Fig. 1). The pre-exposure of PCLs to 10^{-11} M RU did not restore the full CL response probably due to the RU suppressive activity. The pre-treatment with 10^{-8} M RU did not antagonize the HC activity. Finally, pre-treating PCLs with 10^{-5} M RU, and subsequently treating the cells with 10^{-5} M HC, RU exerted a further suppressive activity as shown by the lowest CL response obtained in the experiments ($48 \times 10^3 \pm 16$ CPM). The same results were obtained (data not shown), under the above reported experimental conditions, when PCLs were treated for 1 h with RU and HC in the reaction mixture, or pre-treated for 1 h with RU and then washed before HC treatment.

3.3. Isolation of a GR cDNA from peritoneal cavity leukocytes

A single band of about 468 bp as amplification product from the first PCR was obtained from PCL cDNA using the degenerate primers RT1 and RT2.

To cover the entire transcript sequence, 3′ and 5′ rapid amplification of cDNA ends (3′ and 5′ RACE-PCR) was accomplished; specific primers (D1, S1) designed from the 468 bp fragment were used, and a full-length cDNA of 2592 bp was obtained (Accession No. AY619996).

The complete cDNA sequence presented an open reading frame encoding a protein containing 779 amino acids (Fig. 3) that showed the highest identity (80%) with HbGR1, whereas lower identity degrees with other GRs were found (49% HbGR2a, 48% HbGR2b, 68% RtGR1, 56% RtGR2, and 47% human GRα) (Fig. 3).

PCR amplification of the DlGR1 DNA-binding domain yielded only one product, supporting that the expression of a DNA-binding domain was unique to DlGR1.

3.4. Phylogenetic tree and domains comparison

A phylogenetic tree was constructed by comparing deduced amino acid sequences of various steroid receptors, with DlGR1 and HbGR1 forming a distinguishable cluster included in a multigenic family (Fig. 4). Both the GRs were members of a wider branch inclusive of HbGR2a/b, RtGR1 and RtGR2. Moreover, DlGR1 and HbGR1 appeared to be related to human, mouse, and *Xenopus* GRs.

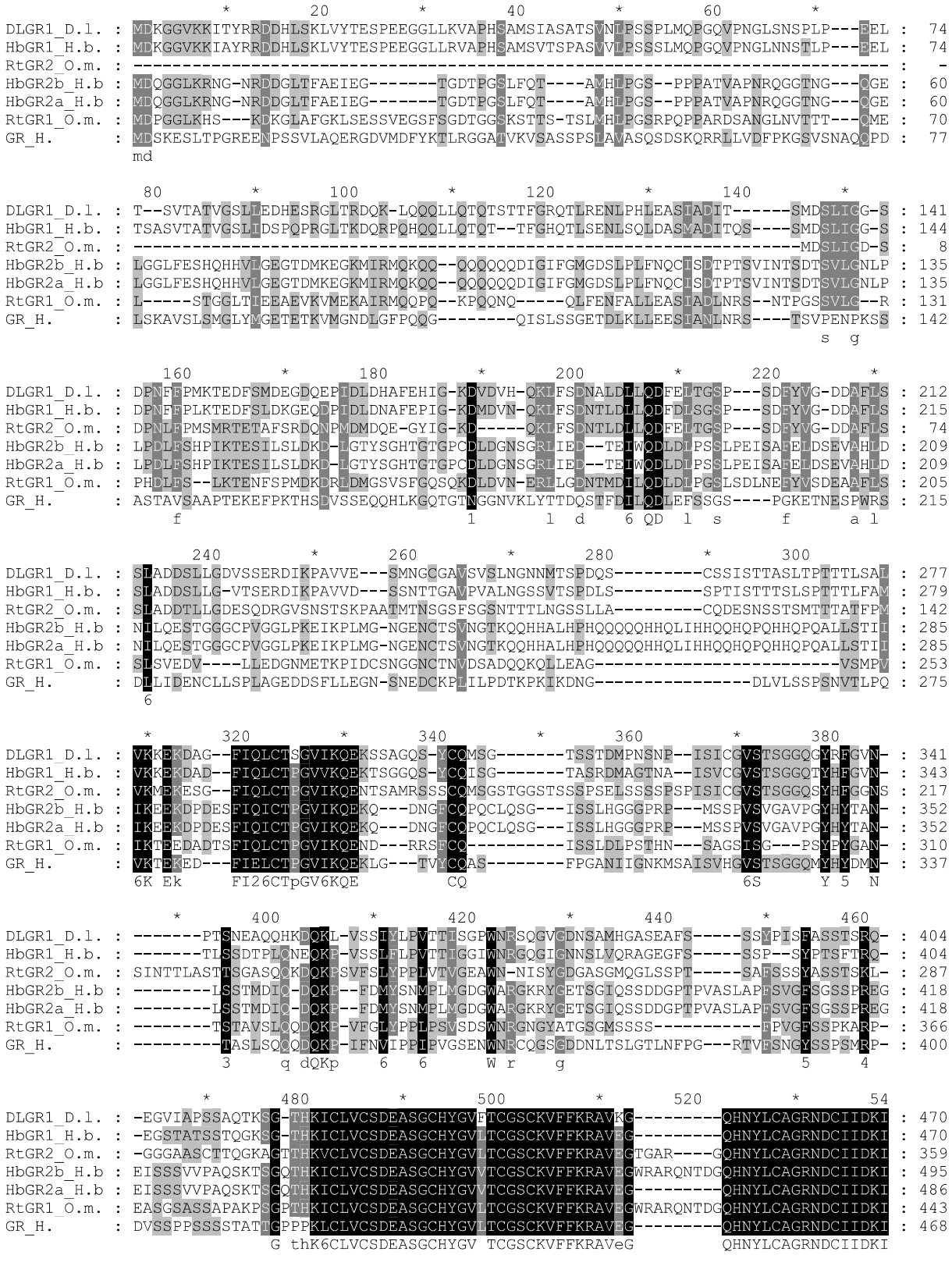

Fig. 3. Deduced amino acid sequence of DlGR1 aligned with the corresponding HbGR1 and HbGR2a/b sequences of *H. burtoni*, RtGR1 and RtGR2 of *Oncorhynchus mykiss*, GR human. Invariant residues are shaded in black, conserved residues in 80% of sequences are shaded in gray with white lettering; conservatively substituted residues in 60% of sequences are shaded in gray with black lettering; *, block of ten nucleotides.

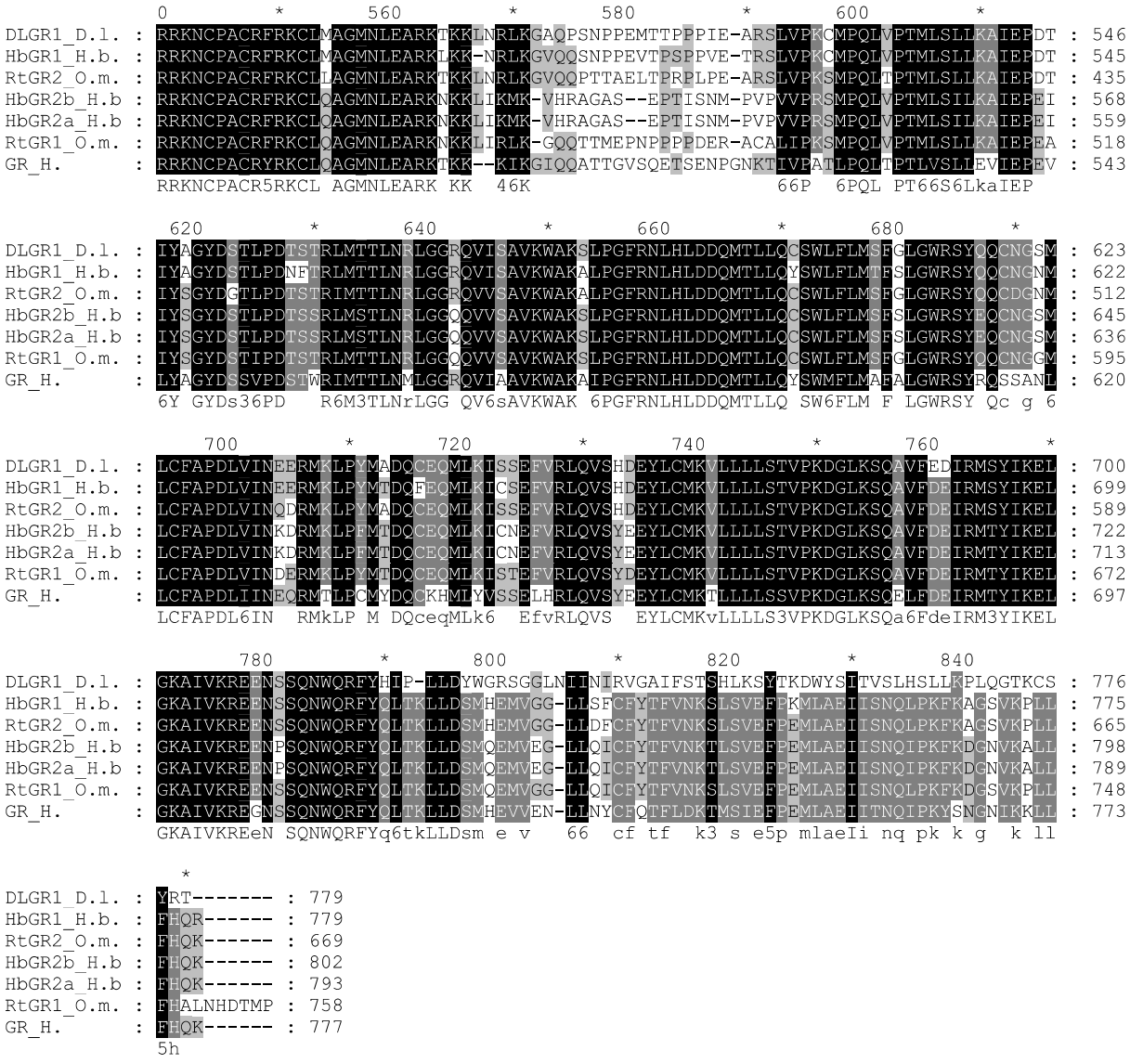

Fig. 3 (*continued*)

Finally, the examined GRs formed a cluster distinct from PR, MR, and AR.

As shown in Table 1, DlGR1 presented four domains including the transcriptional activation domain (aa 1–420), DNA-binding domain (aa 420–485), nuclear localization domain (aa 497–524), and hormone-binding domain (aa 527–779). The sequence of the DNA-binding domain was the most conserved (from 96% identity with HbGR1 to 88% with RtGR2). A high level of homology characterized the hormone-binding domain (from 89% identity with RtGR2 to 74% with hGRα). The sequence of nuclear localization domain presented high levels of identity (from 69% to 88%) with other fish species, whereas a lower level (57%) with the domain of hGRα was observed. The lowest identity values (from 27% to 50%) were found comparing DlGR1 transcriptional activation domain with the corresponding sequences of HbGR2a/b, RtGR1, RtGR2, and hGRα, whereas a high homology level (73%) was found with HbGR1.

3.5. In situ hybridization experiments

DlGR1 transcripts were detected in PCL preparations (Figs. 5a and b). Macrophages, neutrophils, and eosinophil granulocytes were identified in cell preparations stained with Giemsa after the treatment with the riboprobe. A great number of macrophages and, at less extent, neutrophils expressed DlGR1 mRNA, whereas eosinophils were always negative. No signal was observed in PCLs treated with the sense DlGR1 DIG-riboprobe.

Although differential counts were not performed, the above reported findings on the frequency of positive leukocyte types were supported by assaying the probe on PCL populations mainly enriched in Band 1 (macrophages and neutrophils) or Band 2 (eosinophil granulocytes) separated through a continuous Percoll density gradient.

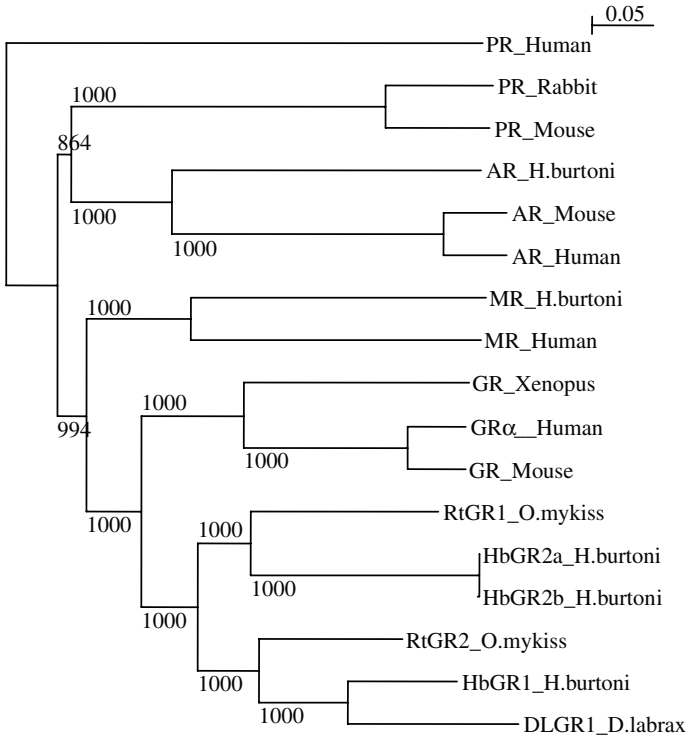

Fig. 4. Phylogenetic tree of steroid receptors including mineralcorticoid receptors (MR), androgen receptors (AR), progesterone receptors (PR), and glucocorticoid receptors (GR). This tree was constructed by the Neighbor-Joining method and bootstrap analysis. Numbers represent the percentages of 1000 bootstrap replicates in which the same internal branch was recovered. The horizontal bar indicates the number of nucleotide substitutions for site. The aligned sequences and their respective Genbank Accession Nos. were: *H. burtoni* HbGR1 AAM27887, *H. burtoni* HbGR2a AAM27888, *H. burtoni* HbGR2b AAM27889, *O. mykiss* GR P49843, human GR P04150, mouse GR P06537, *X. laevis* GR P49844, PR human P06401, PR Rabbit P06186, PR mouse NM008829, *H. burtoni* MR AAM27890, MR human AJ315514, *H. burtoni* AR AY082342, AR mouse NP038504, AR human P10275. The alignment of DlGR1, HbGr1 and RtGR1 and RtGR2 of *Oncorhynchus mykiss*, *Xenopus laevis* and human GR was produced with ClustalX v.1.81 and similarity shaded with GeneDoc v.2.6.002.

Table 1
Identity percentages of *Dicentrarchus labrax* DlGR1 domains with the corresponding sequences from fish, and human GRs

DlGR1 domain	HbGR1 779 aa	HbGR2a 793 aa	HbGR2b 802 aa	RtGR1 758 aa	RtGR2 669 aa	hGRα 777 aa
Transcriptional activation	73	27	27	35	50	28
DNA-binding	96	96	95	93	88	93
Nuclear localization	88	69	69	77	81	57
Hormone-binding	87	86	86	87	89	74

DlGR1, *Dicentrarchus labrax*; HbGR1, HbGR2a/b, *Haplocromis burtoni*; RtGR1, RtGR2, *Oncorhynchus mykiss*; hGR, human.

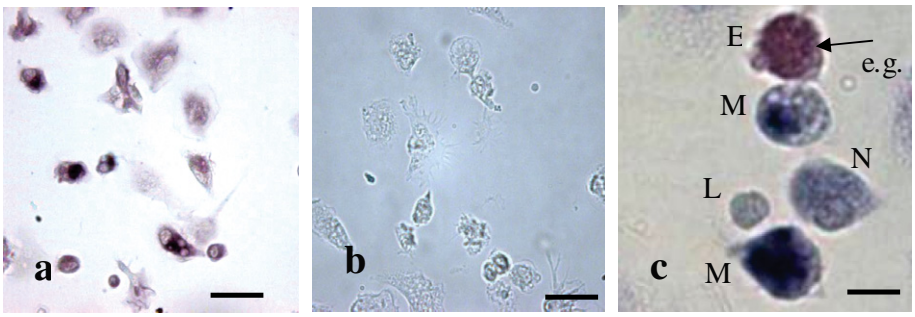

Fig. 5. Location of DlGR1 mRNA in leukocytes separated from the peritoneal cavity. *In situ* hybridization with single-strand type DlGR1 DIG-riboprobe: (a) leukocytes separated from the peritoneal cavity with antisense DlGR1 DIG_riboprobe; (b) control: leukocytes with sense DlGR1 DIG_riboprobe; (a and b): bar = 10 μm; (c) leukocytes treated with DlGR1 antisense DIG-riboprobe and, then, stained with Giemsa. Bar, 40 μm; E, eosinophil granulocyte; M, macrophage; N, neutrophil; L, lymphocyte; e.g., eosinophils granules.

4. Discussion

It is well documented that in fish, cortisol is the major stress-related hormone that modulates both innate and adaptive immune responses. Several papers have been published on the cortisol modulation of fish phagocytes with contrasting results about the respiratory burst response. Stave and Roberson (1985) reported that cortisol reduced, in a dose-dependent fashion, the normal ability of striped bass phagocytes to generate a chemiluminescence response, whereas Wang and Belosevic (1995) showed that cortisol did not affect respiratory burst activity in goldfish macrophages, as measured by the NBT reduction assay.

In a previous paper (Vazzana et al., 2002), we showed that increased cortisol plasma level (700–1000 ng/ml) did not significantly affect CL of stressed sea bass head kidney leukocytes, whereas the CL response of PCLs was not assayed. Vazzana et al. (2003) established that, macrophages and neutrophils from peritoneal cavity of not-stressed sea bass were phagocytes sensitive to zymosan activation, as revealed by an enhanced CL, and macrophages appeared to be more active in ingesting yeast. In the present research, PCLs were treated *in vitro* with three distinct hydrocortisone concentrations before zymosan activation. A significant dose-dependent suppressive effect of 10^{-6} and 10^{-5} M HC on CL was manifest ($P < 0.05$ and $P < 0.01$, respectively) indicating that cortisol modulate peritoneal cavity phagocyte activity. However, 1 h incubation of the cells with each HC concentrations did not abolish CL activity, suggesting that, in part, it could be independent from the hormone-modulation. The possibility exists that this response can be abolished by a more prolonged treatment or higher HC doses. Different effects of cortisol phisiological or pharmacological *in vitro* doses on phagocytes, as reported by other authors (Esteban et al., 2004; Law et al., 2001), could depend on the used fish species, tissue or organ examined, and assays used for revealing modulation of the phagocyte activity.

When PCLs were pre-treated for 1 h with a low mifepristone concentration (10^{-11} M) they became partially insensitive to the action of 10^{-5} M HC supporting a prompt GR-cortisol interaction through a classical genomic pathway. The not-full recovery of CL leukocyte response, could be dependent on a weak suppressive effect of the used RU concentration (10^{-11} M, see below), and/or on a HC action *via* a non genomic pathway insensitive to RU. As regards this, Long et al. (2005) reported a rapid (within 20 min) non-genomic effect of corticosterone on phagocytosis and superoxide anion production by mouse peritoneal macrophages, not inhibited by a brief pre-treatment (5 min) with RU486, suggesting a pathway mediated by presumed membrane-bound receptors. Differently, in *D. labrax*, we registered that, after one hour pre-treatment with RU486, the cells became significantly less sensitive to the subsequent hydroxicorticosterone treatment. A higher RU concentration (10^{-8} M) in the presence of HC caused a weak (not significant) suppressive effect that reach its maximum at the highest RU concentration (10^{-5} M) abolishing the CL response. In addition, when RU alone was tested at the highest concentration (10^{-5} M), the CL level was significantly decreased suggesting that RU may have dose-dependent antagonistic/suppressive activity. These results are in accordance with the *in vitro* differential effects of RU on lyzard splenic macrophages: RU acted as antagonist at 10^{-11} and 10^{-13} M whereas suppressed phagocytic activity at 10^{-7} and 10^{-9} M (Mondal and Rai, 2002). Moreover, the effects of RU treatments on thymidine incorporation of human lymphocytes were antagonistic at 10^{-7} and 10^{-8} M RU, whereas were depressive at 10^{-5} M in the presence or absence of HC (Van Voorhis et al., 1989). Present results did not allow to clarify the basis for a such RU behaviour.

To support that cortisol modulation of CL response could be dependent on HC–GR interactions, a cDNA was isolated and sequenced from PCLs, identified as a GR, and named DlGR1. The sequence appeared to be homologue to the known fish GR cDNA. In particular, we used the procedure and primers reported by Greenwood et al. (2003), and obtained a deduced amino acid sequence which presented the highest homology (80% identity) with the *H. burtoni* HbGR1 and, at less extent (48/49% identity), to HbGR2a/b isoforms. The sequence diversity between DlGR1 and HbGR2 appeared to be at the same order than that observed between HbGR1 and HbGR2. According Greenwood et al. (2003) PCR amplification of the DNA-binding domain excluded the possibility that DlGR1 splice variants could be expressed.

High sequence homology was observed within vertebrate steroid-binding or DNA-binding domains (Evans, 1988). Comparison of DlGR1 presumptive functional domains with those of fish and human GRs displayed that both DNA and hormone-binding domains contained highly conserved sequences to bind EGC nucleotide or hormone molecular structures. The low identity grade observed in the alignments of transcriptional activation domains or nuclear localization domains could be referred to species-specific gene differences. According to Greenwood et al. (2003) and Bury et al. (2003), the resulting phylogenetic tree suggested that fish GRs belong to a multigenic family, and indicated that gene duplication is probably common to teleost fish GRs.

A riboprobe specific to the transcriptional activation domain displayed that DlGR1 could be expressed by peNSritoneal cavity leucocytes. *In situ* hybridization experiments, followed by Giemsa staining, supported that DlGR1 mRNA was mostly exhibited by numerous mNSacrophages and, to a lesser extent, by neutrophils. Eosinophil granulocytes never presented DlGR1-mRNA. To explain the suppressive *in vivo* effect of cortisol on cytotoxic activity of EGLs from stressed sea bass as reported by Vazzana et al. (2002), an alternative modulation mechanism could be invoked. In fact, it is known that GCs inhibit cytokine-mediated eosinophil survival, reduce tissue and circulating eosinophil leukocyte amount, inhibit elements of cytokine "priming" of

eosinophils (Lamas et al., 1991), and, finally, a non genomic *in vitro* effect cannot be excluded (Long et al., 2005). Research are in progress to identify if more genes are present and expressed in sea bass tissues, and the role of DlGR1 in binding several corticosteroid hormones.

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