Expression of a glucocorticoid receptor (DlGR1) in several tissues of the teleost fish *Dicentrarchus labrax*

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Abstract

Since glucocorticoids have a role in maintaining the homeostatic status in fish, in the present paper mRNA expression (*in situ* hybridization) and tissue immunohistochemical localization of a glucocorticoid receptor (DlGR1) in several *Dicentrarchus labrax* organs are reported. Riboprobe and specific antibodies were prepared by using the DlGR1 that has been previously cloned and sequenced from peritoneal cavity leukocytes. Both mRNA and receptor were identified in head kidney, spleen, gills, intestine, heart and liver tissues. The functional roles of DlGR1 localization are discussed.
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1. Introduction

Stressful environmental conditions exert deleterious effects on many physiological functions of fishes by acting on the neuroendocrine system (Wendelaar-Bonga, 1997; Mommsen et al., 1999). The resulting increased levels of serum corticosteroids and catecholamines (Mazeaud and Mazeaud, 1981; Vazzana et al., 2002) restore the normal functions (McEwen and Brinton, 1987). Cortisol is the glucocorticoid that virtually acts on all tissues and affects a wide variety of biological responses including control of intermediary metabolism, ionic and osmotic regulation, growth and immune functions (Mommsen et al., 1999; Vazzana et al., 2002). Recently we have shown, through *in vivo* and *in vitro* experiments, that increased cortisol level affects sea bass innate immunity exerting its effect *via* the binding to a cytosolic receptor (DlGR1) (Vizzini et al., 2007).

Glucocorticoid receptors (GR) have been found in gills, kidney, spleen, brain, liver, and intestinal mucosa examined in separated fish species by using various methods (Sandor et al., 1984; Maule and Schreck, 1990; Greenwood et al.,

2003; Tagawa et al., 1997; Knoebl et al., 1996; Allison and Omeljaniuk, 1998; Lee et al., 1992; Chakraborti and Weisbart, 1987; Pottinger et al., 1994; DiBatista et al., 1983). *In situ* hybridization and/or immunocytochemical staining have also been used (Uchida et al., 1998; Takahashi et al., 2006). However, a systematic analysis of several organs from a same species has not been carried out.

Vizzini et al. (2007) cloned and sequenced a 2592 bp cDNA encoding a glucocorticoid receptor (DlGR1) from sea bass (*Dicentrarchus labrax*) peritoneal leukocytes where it can be expressed (*in situ* hybridization assay). The DlGR1 functional domains presented homologies with glucocorticoid receptors of vertebrate species. Dose-dependent cortisol inhibitory effects and significant competitive activity of a receptor blocker (a low concentration of mifepristone RU486) supported that cortisol–GR interaction is involved in modulating phagocytes chemiluminescent response via a genomic pathway.

In this paper, the expression of DlGR1 mRNA was shown by using *in situ* hybridization method, while the GR localization in tissues of several organs was revealed by immunohistochemistry method. In addition, specific antibodies in western blot assay of tissue lysate supernatants supported the presence of DlGR1 in each organ.

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2. Materials and methods

2.1. Fish

Sea bass (200–250 g) were obtained from a commercial fish farm (Trappeto, Italy). Fish were anaesthetized with 0.05% 3-aminobenzoic acid ethyl ester (Sigma–Aldrich, Germany) in seawater, and sampled at the farm. Head kidney, spleen, intestine, gill, heart and liver were surgically removed.

2.2. Tissue homogenate supernatant preparation (THS)

All tissues were quickly frozen with dry ice and stored at $-80\,^{\circ}\mathrm{C}$, until processing. Tissues were crushed in liquid nitrogen, treated on ice for 1 h with 1 ml of lysis buffer (RIPA: 0.5% sodium deoxycholate minimum 97%; 1% NP40; 0.1% SDS with PBS-T (Na_2HPO_4 1 M, NaH_2PO_4 1 M, NaCl 1.5 M, 0.1% Tween 20) supplemented with a protease inhibitor cocktail (Sigma) diluted 1:200. Then the samples were centrifuged at $15.000 \times g$ for 10 min at $4\,^{\circ}\mathrm{C}$, supernatants were collected, dialyzed against 50 mM Trizma base pH 7.5, and total protein content determined according to the Bradford method (Bradford, 1976).

2.3. SDS-PAGE and Western blot

SDS-PAGE (7.5%) under reducing conditions was performed according to the Laemmli method (Laemli, 1970). The protein pattern of THS (25 μg protein content), was transferred to a nitrocellulose membrane by using a semi-dry transfer apparatus (BioRad) and blocked with 5% bovine serum albumin (BSA, Sigma) in TBS-T (20mM Trizma base pH 7.5, 300 mM NaCl, 0.1% (v/v) Tween 20 with 0.02% sodium azide) for 1 h at room temperature (r.t.). To identify DlGR1, the membrane was incubated with specific anti-DlGR1 antiserum (1:1000 dilution, 3 h at r.t.), washed with TBS-T (three times for 5 min), incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (SIGMA, 1:10000 for 1 h at r.t.), and washed with TBS-T (three times for 5 min). Membranes were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (BCIP/NBT Sigma).

2.4. Histological methods

The organs were fixed in Bouin's solution (saturated picric acid:formaldehyde:acetic acid 15:5:1) for 24 h, embedded in paraffin and serially cut at 6 μm (Leica RM2035 microtome).

Sections of each organ were stained with Mallory Trichromic (Mahoney, 1973; Mazzi, 1977). Histological observations were carried out by using a Leica DMRE microscope, and tissue architecture recognized according to Wilson and Laurent (2002); Pfeiffer et al. (1999); Zapata (1979, 1982) and Elbal et al. (2004).

2.5. In situ hybridization with DIG-riboprobe

Digoxigenin-11-UTP-labeled riboprobe (DIG-riboprobe) was prepared as previously reported by Vizzini et al. (2007). According to Le Guellec (1998), tissues sections, washed with PBS-T, were digested with proteinase K (1 μl/ml) (Sigma) in PBS-T, and blocked with stop-solution (2 mg/ml glycine in PBS-T). After washing in PBS-T, the sections were post-fixed with 4% formaldehyde in PBS-T for 30 min, and pre-hybridization with hybridization solution (50% formamide, 50 μg/ml heparin, 500 μg/ml yeast tRNA, 0.1% Tween 20, $5\times$ SSC (SSC: 0.15 M NaCl/0.05 M sodium citrate, pH 7) at $37\,^{\circ}\mathrm{C}$ for 1 h, and hybridization with 15% riboprobe in hybridization solution over night at $37\,^{\circ}\mathrm{C}$.

Sections, rinsed (10 min) in preheated ($37\,^{\circ}\mathrm{C}$) PBS-T, were further rinsed in washing solution (0.3% SSC $20\times$, 1% Tween 20, DW), incubated for 30 min at r.t. with 2% horse serum in PBS-T, and then incubated (1 h at r.t.) with anti-Digoxigenin-Fab-Ab (Roche) diluted 1:100 in the horse serum solution. After washing with PBS-T, the sections were incubated in BCIP/NBT liquid substrate system (Sigma).

Control experiments were run using the corresponding sense cRNAs at the same dilution.

2.6. Immunohistochemistry with polyclonal antibodies

Anti-DlGR1 polyclonal antibody was raised in rabbit by using as antigen a peptide corresponding to the hydrophilic sequence located in the N-terminal region (transcriptional activation domain) of DlGR1 (residues from 85 to 98: LEDHESRGLTRDQK). The peptide sequence was selected by antigen-prediction program and synthesized by Sigma Genosys (UK). The sections, washed in PBS-T, were incubated with 3% BSA (Sigma) in PBS-T for 2 h at r.t., and then with diluted primary antibody (1:500 in PBS-T) overnight at $4\,^{\circ}\mathrm{C}$. Anti-rabbit IgG phosphatase alkaline conjugated antibody was used as secondary antibody (1:10.000 for 1 h and 30 min at r.t.). The sections were rinsed with PBS-T, stained with BCIP/NBT liquid substrate system (Sigma). Controls were performed with the preimmune rabbit IgG at the same dilution.

3. Results

3.1. Western blot

To establish the presence of DlGR1, preliminary immunoblotting assays were performed. Fig. 1 shows that the specific anti-DlGR1 antibody identified a single band of 74 kDa in all the examined tissues. The apparent molecular size was in conformity with the presumptive DlGR1 size calculated from the deduced amino acid sequence.
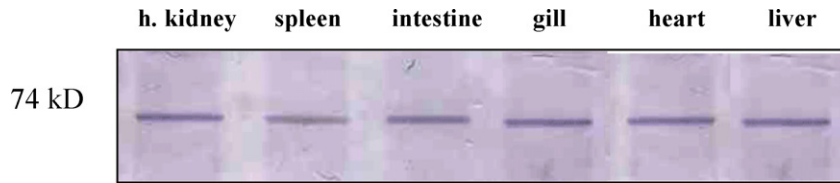
Fig. 1. Western blot analysis, with anti-DlGR1 antibodies, of tissue extracts from several *D. labrax* organs.
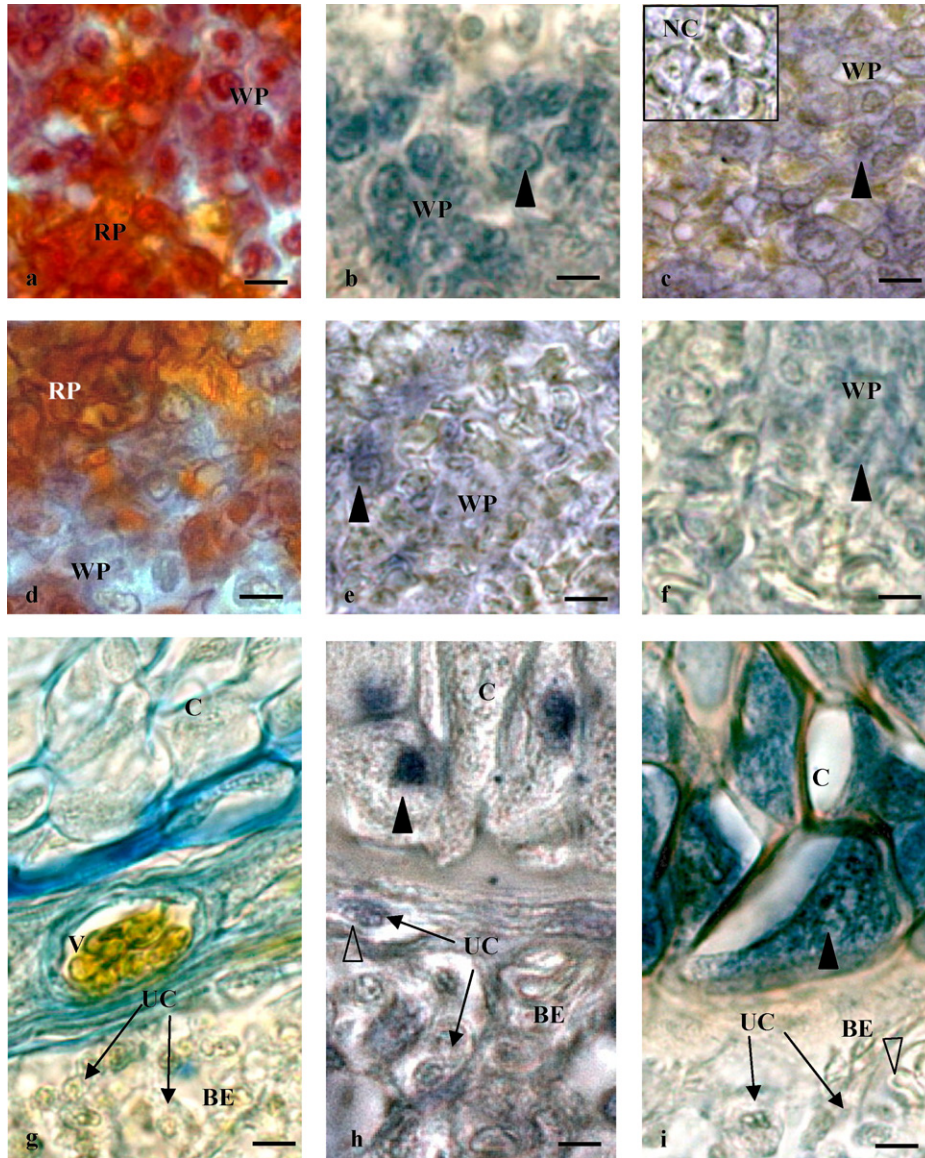

Fig. 2. *In situ* hybridization and immunohistochemistry of *D. labrax* head kidney, spleen, gill histological sections. (a–c) Head kidney transverse sections. (a) Mallory Trichromic stain; (b) localization of DlGR1 mRNA (ISH) in the nucleus and cytoplasm of some cells of parenchyma (black arrow head); (c) DlGR1 protein expression (immunohistochemistry) in the nucleus and cytoplasm of parenchyma cells (black arrow head). WP: white pulp, RP: red pulp, NC: negative control. (d–f) Spleen transverse sections. (d) Mallory Trichromic stain; (e) localization of DlGR1 mRNA (ISH) in the nucleus and cytoplasm of parenchyma cells (black arrow head); (f) DlGR1 protein expression (immunohistochemistry) in the nucleus and cytoplasm of parenchyma cells (black arrow head). WP: white pulp, RP: red pulp. Bar: 20 μm. (g–i) Gill horizontal sections. (g) Mallory Trichromic stain; (h) localization of DlGR1 mRNA (ISH) in the nucleus of hypertrophic zone chondrocytes (black arrow head) and of undifferentiated cells (arrow head); (i) DlGR1 expressed (immunohistochemistry) in the cytoplasm of chondrocytes in the hypertrophic zone (black arrow head) and in the nucleus and cytoplasm of undifferentiated cells (arrow head). C: chondrocytes, UC: undifferentiated cells, V: vessel, BE: branchial epithelium.

3.2. ISH and immunohistochemistry show DlGR1 expressed and localized in head kidney, spleen, gills, intestine, heart and liver tissues

In head kidney transverse section red and white pulp can be distinguished (Fig. 2a). DlGR1 mRNA and protein were present in the nucleus and in the cytoplasm of white pulp cells (Fig. 2b and c).

Spleen section show lymphoid white pulp and red pulp erythropoietic tissue (Fig. 2d). ISH and specific antibody revealed DlGR1 mRNA and protein in the nucleus and cytoplasm of white pulp cells (Fig. 2e and f).

Fig. 2g and i show gill filament horizontal sections. Mallory Trichromic displayed chondrocytes in the hypertrophic zone surrounded by connective tissue, a vessel containing erythrocytes, and branchial epithelium with undifferentiated cells (Fig. 2g). *In situ* hybridization signal was located in the nucleus of branchial epithelium undifferentiated cells and chondrocytes (Fig. 2h). Antibody identified DlGR1 in the

nucleus and cytoplasm of undifferentiated cells and in the cytoplasm of chondrocytes (Fig. 2i).

In intestine transverse section the intestinal mucosa with the brush border turned to the intestinal lumen, and the columnar cells intermingled with mucous cells were distinguished (Fig. 3a). DlGR1 mRNA was expressed in the nucleus (Fig. 3b), and the antibody identified the DlGR1 in the cytosol of the columnar cells (Fig. 3c).

In heart transverse section striate muscular fibres and the circulating erythrocytes were recognized (Fig. 3d). The transcript was seen in the nucleus of the muscular fibres (Fig. 3e), whereas DlGR1 was identified in their cytosol (Fig. 3f).

In liver transverse section hepatocytes and parenchyma tissue were seen with Mallory Trichromic stain (Fig. 3g), mRNA was found in the hepatocytes nucleus (Fig. 3h) and DlGR1 was in the cytoplasm (Fig. 3i).

In all the sections, no signal was found when the specific riboprobe was replaced with the complementary oligonucleotide probe. Immunohistochemical reaction was not found
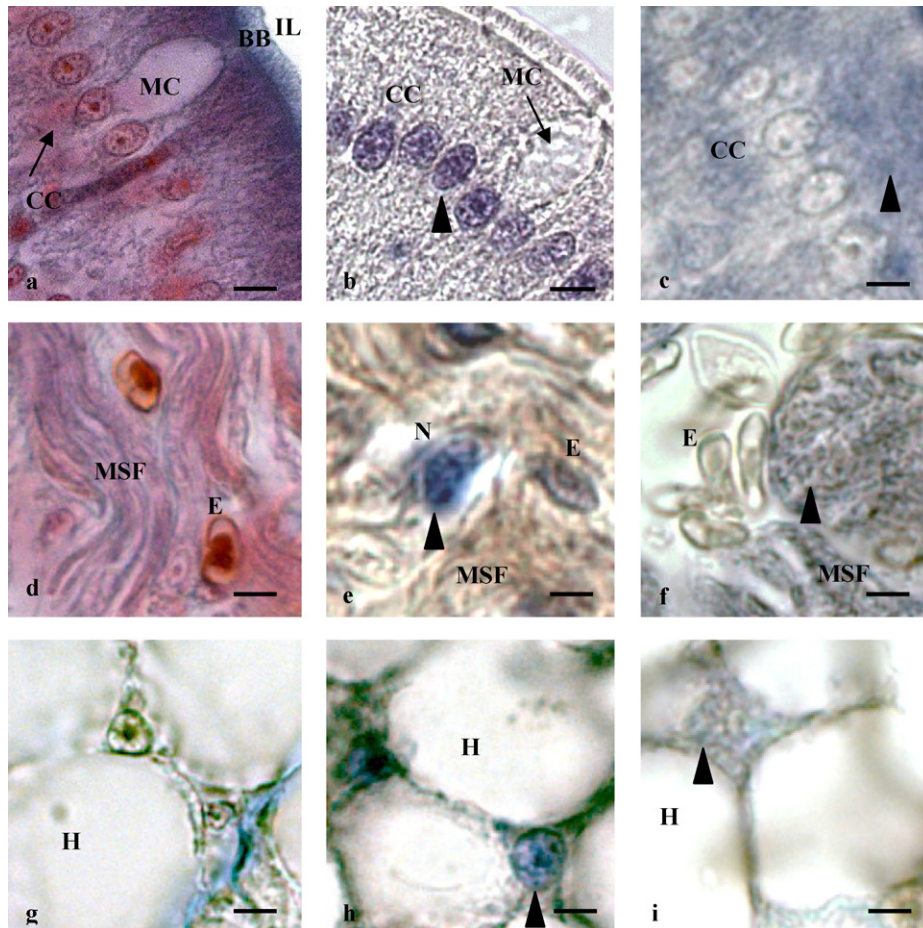

Fig. 3. *In situ* hybridization and immunohistochemistry of *D. labrax* intestine, heart, liver histological sections. (a–c) Intestine transverse sections. (a) Mallory Trichromic stain; (b) cellular localization of DlGR1 mRNA (ISH) in the nucleus of columnar cells; (c) DlGR1 protein expressed (immunohistochemistry) in columnar cell cytoplasm. CC: columnar cells, MC: mucous cell, IL: intestinal lumen, BB: brush border. (d–f) Heart transverse sections. (d) Mallory Trichromic stain; (e) localization of DlGR1 mRNA (ISH) in the muscular striate fibres nucleus; (f) DlGR1 protein expressed (immunohistochemistry) in the cytoplasm of muscular striate fibres. MSF: muscular striate fibres, E: erythrocytes, N: nucleus of muscular striate fibres. (g–i) Liver transverse sections. (g) Mallory Trichromic stain; (h) localization of DlGR1 mRNA (ISH) in the hepatocytes nucleus; (i) DlGR1 protein expressed (immunohistochemistry) in the cytoplasm of the hepatocytes. H: hepatocyte. Bar 20 μm.

when primary antibody was omitted as well as when the specific antibody was substituted with preimmune serum (data not shown).

4. Discussion

In this study we show the distribution of *Dicentrarchus labrax* DlGR1 expressed in several organs supporting the wide physiological role of cortisol that is the main glucocorticoid hormone, responsible for fish homeostasis maintenance. Accordingly, the transcript and the DlGR1 protein were expressed in all the examined tissues including head kidney, spleen, gills, intestine, heart and liver. DlGR1 mRNA was localized in the nucleus and cytoplasm of spleen and head kidney white pulp cells, in the nucleus of branchial epithelium undifferentiated cells and chondrocytes, of intestinal columnar cells, of cardiac muscular fibres, of hepatocytes. The DlGR1 protein was in the nucleus and cytoplasm of head kidney and spleen white pulp cells, of branchial epithelium undifferentiated cells and in the cytoplasm of gills chondrocytes, of muscular striate fibres and of hepatocytes. The cytolocalization of mRNA and protein suggests various phases of the cellular activity. The transcript is mainly present in the nucleus where it's synthesized and its dislocation toward the cytoplasm occurs for the transduction. In the nucleus the presence of ligand-bound DlGR1 is due to its transcriptional regulation activity, whereas unliganded GR is mainly localized in the cytoplasm (Teitsma et al., 1998; Bury and Sturm, 2007).

Although it's difficult to distinguish in head kidney and spleen histological sections, treated for ISH and immunohistochemistry, the red and white pulp, it's known that macrophages and neutrophils, which are components of the white pulp, expressed DlGR1, as shown in peritoneal cavity leukocytes of sea bass (Vizzini et al., 2007).

In the spleen that is rich in free monocytes or macrophages (Zapata, 1982), DlGR1 transcript and protein were expressed in white pulp cells found in a wide zone around melanomacrophage centres. The presence of GR in head kidney and spleen parenchyma is supported by binding studies with [^{3}H] cortisol carried out in coho salmon (Maule and Schreck, 1990). The expression of DlGR1 in the head kidney and spleen parenchyma shows the important role of GC on the immune response, particularly in the immuno-suppression of lymphocytes and macrophages of these lymphoid organs (Verburg-van Kemenade et al., 1999). Moreover cortisol has a rapid effect on head kidney (Stave and Roberson, 1985; Vazzana et al., 2002) and peritoneal cavity leukocytes phagocytic activity (Vizzini et al., 2007), inhibiting the respiratory burst activity, as well as the total peroxidase content of leukocytes (Esteban et al., 2004).

In the gills, cortisol plays an important role in osmoregulatory adaptation, stimulating proliferation and differentiation of the ion-transporting cells (chloride cells) as well as regulating the Na$^+$/K$^+$-ATPase pump (McCormick, 1990, 1995;

Flick and Perry, 1989; Seidelin et al., 1999). DlGR1 mRNA and protein location in the nucleus of undifferentiated cells supports cortisol may regulate the functional differentiation of the branchial epithelium, pavement and chloride cell types as shown in chum salmon fry (Uchida et al., 1998). Moreover, the protein was expressed in the nucleus of cartilaginous cells and in the cytoplasm of undifferentiated cells.

Fish intestine, like the gills, is a major osmoregulatory organ. Cortisol, by glucocorticoid and mineralcorticoid receptors, has a key regulatory role in fish osmoregulation (Prunet et al., 2006), promoting intestinal ion and water absorption (Karnaky, 1998; Loretz, 1995; Collie and Hirano, 1987), and the seawater adaptation of euryhaline teleosts, with a significant rise in GR mRNA in the intestine (Takahashi et al., 2006). Therefore DlGR1 mRNA and protein expression, respectively, in the nucleus and cytosol of the columnar cells, can be related to the osmoregulatory function.

In accordance with the glucocorticoids role in the cardiovascular function (Yagil and Krakoff, 1988), DlGR1 was found to be expressed in the nucleus of cardiac muscular fibres and the receptor was located in the cytoplasm of these fibres. As well as mineralcorticoids, glucocorticoids exert potent influences on arterial pressure (Yagil and Krakoff, 1988), and Van den Berg et al. (1990) reported that the cortisol antagonist RU38486, by acting on GR, increased the systolic blood pressure.

The finding that DlGR1 mRNA was expressed in the nucleus of the hepatocytes, whereas the protein was contained in the cytosol of these cells, supports the action of the hormone in modulating the hepatic glucose metabolism (Mommsen et al., 1999).

In conclusion, the presence of a glucocorticoid receptor in all the organs here examined supports that, in *D. labrax*, the genomic pathway is involved in cortisol-dependent homeostasis of the organism. Research is in progress to examine DlGR1 expression in the gonads at the various developing stages as well as in the brain.

Acknowledgments

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