Neuroscience 132 (2005) 665–672

CYTOCHROME-C OXIDASE IS ONE OF SEVERAL GENES ELEVATED IN MARGINAL RETINA OF THE CHICK EMBRYO

L. E. PARAOANU,* B. WEIß, A. A. ROBITZKI AND P. G. LAYER

Developmental Biology and Neurogenetics, Darmstadt University of Technology, Schnittspahnstrasse 3, D-64287 Darmstadt, Germany

Abstract—The retinal ciliary margin is particularly relevant for the correct generation and regeneration of vertebrate retinae, since pluripotent stem cells are located there throughout development, and—at least in some species—even until adult stages. Our aim was to identify factors (genes) which are involved in processes of proliferation and differentiation in the developing chicken retina. Reverse transcription–polymerase chain reaction differential display was used to identify genes that were differentially expressed in chick central and peripheral embryonic retina. Candidate genes analyzed through sequencing and database searches were confirmed by Northern blot analysis and histochemistry. A series of differentially expressed genes were detected, including a neuronal cell adhesion molecule, an esterase, and homeobox gene products. One of the sequenced products was identified as subunit I of cytochrome-c oxidase (COX-1), an enzyme which is central to energy metabolism and particularly relevant for developing nervous systems. Northern blot analysis confirmed its up-regulation in the chick peripheral retina, being maximal at embryonic day 7. In the retinal pigmented epithelium its expression is lower than in the retinal periphery but higher than in central retina. COX histochemistry revealed distinct laminar patterns in central retina, but also an elevated level of activity in the peripheral retina throughout development. These data not only show that the developing ciliary margin of the chick retina has high energy requirements, but also indicate that COX-1 could play essential roles in developing cells and in stem cells of the eye periphery.

Key words: cytochrome c-oxidase, ciliary margin, differential display, cell proliferation, retinal development, stem cells.

The developing retinal neuroepithelium contains multipotential precursor cells that give rise to all of the neurons and the glial cell types present in the adult retina. The generation of such a highly organized laminar structure requires coordinated events of cell proliferation, migration and differentiation. In the chicken retina, the permanent withdrawal of individual cells from the mitotic cycle begins on embryonic day 3 (E3), and continues until E8 in the central retina and for a few more days at the periphery (Adler, 2000). Thus, retinal differentiation follows a spatio-temporal gradient from the center to the periphery (Layer and Kotz, 1983; Prada et al., 1999). The formation of a stratified structure begins at E6 and ends at E14 (Spence and Robson, 1989). The chicken retina shows also a higher differentiation level at early developmental stages compared with the whole organism. In any given retinal area, the first cells leaving the cycle are determined to become ganglion cells, and the last ones bipolar cells (Prada et al., 1991). However, all classes of neurons, at varying proportions, are being produced during most of the time. Fischer and Reh (2000) have found that new neurons are added to the retina of the chicken via proliferation and subsequent differentiation of neurons and glia at the retinal margin in a zone highly reminiscent of the ciliary marginal zone of lower vertebrates, e.g. fish and amphibians. The ciliary marginal zone is represented by a circumferential zone of cells that generates new retinal cells which are continually added at the anterior margin of the retina. The regenerative capacity of the ciliary margin in the chick embryo was clearly demonstrated by the reconstitution of organized laminar reaggregates from dispersed cells derived from the retinal periphery (Layer et al., 1990), which included pluripotent embryonic stem cells (Willbold and Layer, 1992). Most interestingly, stem cells still exist in the adult eye periphery of chicken (Fischer and Reh, 2000) and mice (Ahmad et al., 2000, 2004; Tropepe et al., 2000), stressing the significance of this tissue part for the topic of retinal regeneration.

Patterning and differentiation within the retina is regulated by cell intrinsic as well as cell extrinsic mechanisms. This is reflected by differential expression of diverse genes, as has been demonstrated repeatedly, e.g. by microarray and *in situ* analyses for the chick retina (Hackam et al., 2003). In order to better understand the molecular basis of tissues that are still proliferative, e.g. as the ciliary retinal margin, or of tissues which are undergoing differentiation processes, like the more central retina, we have compared gene expression patterns of central and peripheral retinae by differential display–reverse transcription–polymerase chain reaction (DD-RT-PCR). Besides a multitude of other gene products, one of the genes up-regulated in the peripheral retina was the cytochrome-c oxidase I (COX-1; ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1). In mature nervous tissues, which require much energy, this enzyme is an indicator of neuronal activity (Wong-Riley, 1989). Here, our genetic as

*Corresponding author. Tel: +49-6151-166105; fax: +49-6151-166548.
E-mail address: paraoanu@bio.tu-darmstadt.de (L. E. Paraoanu).

Abbreviations: cDNA, complementary DNA; COX-1, cytochrome-c oxidase I; DD-RT-PCR, differential display–reverse transcription–polymerase chain reaction; DIG, digoxigenin; E, embryonic day; EST, expressed sequence tag; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; N-CAM, neuronal cell adhesion molecule; ONL, outer nuclear layer; OPL, outer plexiform layer; PBS, phosphate-buffered saline; RPE, retinal pigment epithelium; SSC, standard saline citrate.

0306-4522/05$30.00+0.00 © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2004.11.055

well as histochemical data reveal pronounced expression of COX-1 during development of the chick retina, particularly high in the marginal retinal periphery. The possible functional implications of COX-1 for retinal development are briefly discussed.

EXPERIMENTAL PROCEDURES

Preparation of chicken retina

All experiments were carried out in accordance with the German and European Union law guidelines for the care and use of laboratory animals. Care was taken to minimize the number of embryos used and their suffering.

The retinal tissue was isolated from approximately 300 chick embryos at different embryonic stages (White Leghorn; *Gallus gallus domesticus*). Fertilized chicken eggs were purchased from a local hatchery and incubated at 38 °C for the desired period with occasional turning. Such embryos grow normally and correspond to the stages of Hamburger and Hamilton (1951) at given Es of ontogenesis. Thus, embryonic stages will be referred to throughout this paper. The embryos were decapitated and the eyes were carefully removed by tearing off the attached connective tissue and soaked on ice-cold 0.1 M phosphate-buffered saline, pH 7.4 (PBS). To isolate the embryonic day 6 (E6) retinal RNA necessary for DD-RT-PCR and E5, E6, E7, E8 retinal RNA necessary for Northern blot analysis, the eye was sectioned with scissors along the ora serrata in order to separate the central and the peripheral retina (see Fig. 1). The lens and the vitreous body were removed and the central retina was collected. At the eye periphery, the retina and the pigment epithelium are strongly connected. Attention was taken to avoid contamination with retinal pigmented epithelial cells. For isolating the RNA, the central and peripheral parts were homogenized in Lysis-Buffer RLT (Qiagen, Hilden, Germany; RNeasy kit). For histochemistry, the eyes were washed in PBS for 5 min and immersed in fixative.

Isolation of total RNA

Fifty milligrams tissue (wet weight) was collected in 350 μl RLT Lysis-Buffer (Qiagen) including freshly added 10 μl mercaptoethanol. The tissue was homogenized by repeatedly passing through a sterile pipette tip and then centrifuged at 14,000 r.p.m. for 3 min; 350 μl of retina lysate were mixed with an equal volume of 70% ethanol. Total RNA was extracted with a RNA isolation kit (RNeasy kit; Qiagen) and digested with DNase-I (Boehringer, Mannheim, Germany).

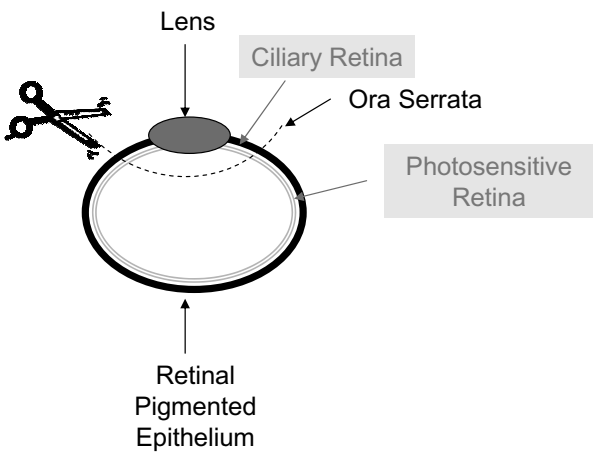

Fig. 1. Scheme of isolation of central or peripheral retinal tissues.

DD-RT-PCR

Differential display has been developed as a tool for the comparison, identification, and isolation of genes expressed as mRNA in various cells under designated conditions. We performed the DD-RT-PCR in order to identify genes that are differentially expressed in the central and peripheral chicken retina at E6 (Liang and Pardee, 1992). The reverse transcription reaction was performed using 3 μg of total retina RNA, 5 mM $MgCl_2$, 1 mM dATP, dCTP, dTTP, dGTP, 1 U/μl RNasin, 5 μM downstream primer-GeneExScreen oligo $(dT)_{11}N$ (Biometra, Göttingen, Germany) and 45 U AMV-Reverse transcriptase (Promega, Mannheim, Germany) in an end volume of 20 μl. The complementary DNA (cDNA) was synthesized by incubating the reaction mix for 1 h at 42 °C and stopped by incubation for 5 min at 95 °C. An aliquot (27 μl) of the cDNA sample was then added to a PCR solution master mix containing 100 μM dNTPs, 25 U of "Prime Zyme" DNA-polymerase (Biometra), 25 μM "downstream Primer" (T12MN) and 2 μM arbitrary "upstream Primers" U^1–U^{26} (GeneExScreen Primer; Biometra). Twenty-six different arbitrary upstream primers were used in combination with the appropriate downstream primer from the "GeneExScreen Primer kit" (Biometra). The PCR parameters were: 95 °C for 4 min, followed by 94 °C for 40 s, 40 °C for 1 min, 72 °C for 2 min with 40 cycles, then 72 °C for 5 min for elongation. The PCR reactions were performed in a thermocycler UNO-thermoblock 96 (Biometra). The PCR amplification products were analyzed by electrophoresis in denaturing 6% polyacrylamide gels at 40–60 V/cm. Nucleic acids were stained by using an improved silver staining method (Bassam et al., 1991). The cDNA bands that showed different expression patterns in the central and peripheral retina were cut from the gel and transferred to microfuge tubes. The gel band was incubated with 100 μl H_20 for 1–12 h at 37 °C. The obtained cDNA fragments were re-amplified using the same primer set and PCR conditions used for the differential display reactions. The re-amplified DNA fragments were subcloned to pSVK3 vector (Pharmacia-Biotech, Freiburg, Germany) and sequenced. The amplimers were sequenced using a 4 μl plasmid DNA, 8 μl terminator ready reaction mix (ABI PRISM Ready Reaction DyeDeoxy terminator cycle sequencing kit with AMpliTaq FS; Applied Biosystems, Weiterstadt, Germany), 4 pmol T7 promoter primer oligonucleotide 5′TAATACGACTCACTATAGGGAGA3′ (AGS, Heidelberg, Germany) and H_2O to a final volume of 20 μl. The amplified DNA was dried and solved in 10 μl template suppression reagent (P/N 401674), incubated for 2 min at 80 °C and used for the capillary electrophoresis in the DNA-Analyze-System ABI PRISM 310 (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). The obtained DNA sequences were analyzed using the BLAST program and the GenBank database. For further details see Weiß (1997).

Northern blot analysis

Northern hybridization analysis was performed using a RNA blot containing central and peripheral retina RNA. Ten micrograms total RNA from each probe were electrophoresed through a 1% agarose gel containing 6% formaldehyde, transferred to a Hybond-N^+ nylon membrane (Amersham Buchler, Braunschweig, Germany) and used for the hybridization. After transfer, the nucleic acids were cross-linked to the membrane in a UV cross-linker. The membranes were pre-hybridized in buffer containing 50% formamide, 5× standard saline citrate (SSC; 3 M NaCl, 0.3 M trisodium citrate), 50 mM sodium phosphate buffer, pH 7, 2% block solution, 0.1% N-lauroylsarkosin, 7% sodium dodecyl sulfate for 6 h at 50 °C and then hybridized at 50 °C in fresh buffer. The hybridization buffer contained 10 ng of a 140 bp fragment of the chicken COX-1. The cDNA probe was labeled using random primed labeling method (Feinberg and Vogelstein, 1983) and a digoxigenin (DIG)-High-Prime labeling Mix (Boehringer, Mannheim, Germany). A β-actin probe (0.7 kb) was used to determine the relative amount of RNA in each lane. Membranes were washed in 2×2 SSC, 2×0.5 SSC, 1×2 SSC for varying times. The