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A crystallin gene network in the mouse retina

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ABSTRACT

The present study was designed to examine the regulation of crystallin genes and protein in the mouse retina using the BXD recombinant inbred (RI) strains. Illumina Sentrix BeadChip Arrays (MouseWG-6v2) were used to analyze mRNA levels in 75 BXD RI strains along with the parental strains (C57Bl/6J and DBA/2J), and the reciprocal crosses in the Hamilton Eye Institute (HEI) Retina Dataset (www.genenetwork.org). Protein levels were investigated using immunoblots to quantify levels of proteins and indirect immunohistochemistry to define the distribution of protein. Algorithms in the Genomatix program were used to identify transcription factor binding sites common to the regulatory sequences in the 5′ regions of co-regulated set of crystallin and other genes as compared to a set of control genes. As subset of genes, including many encoding lens crystallins is part of a tightly co-regulated network that is active in the retina. Expression of this crystallin network appears to be binary in nature, being expressed either at relatively low levels or being highly upregulated. Relative to a control set of genes, the 5′ regulatory sequences of the crystallin network genes show an increased frequency of a set of common transcription factor-binding sites, the most common being those of the Maf family. Chromatin immunoprecipitation of human lens epithelial cells (HLEC) and rat retinal ganglion cells (RGC) confirmed the functionality of these sites, showing that MafA binds the predicted sites of CRYGA and CRYGD in HLE and CRYAB, CRYGA, CRYBA1, and CRYBB3 in RGC cells. In the retina there is a highly correlated group of genes containing many members of the α- β- and γ-crystallin families. These genes can be dramatically upregulated in the retina. One transcription factor that appears to be involved in this coordinated expression is the MAF family transcription of factors associated with both lens and extralenticular expression of crystallin genes.

1. Introduction

The crystallins are the major structural proteins of the eye's lens, making up 90% of the soluble proteins. They consist of the ubiquitous crystallins, which comprise three major classes: α, β, and γ, in addition to a group of taxon-specific crystallins, also called enzyme-crystallins since they were largely recruited from genes encoding housekeeping enzymes (Wistow and Piatigorsky, 1988). Time has blurred the distinction between the ubiquitous and enzyme-crystallins. Initially, the ubiquitous crystallins were recognized as the primary component of the vertebrate lens, lending the lens its optical properties of transparency and refractive focusing of light. Although initially they were thought of as being solely lens proteins, it is now known that the crystallins are expressed in other tissues such as the brain, heart, kidney, lungs, and retina (Clayton et al., 1986; Head et al., 1991; Smolich et al., 1994). The α-crystallin proteins are the most studied, and have a high degree of homology with small heat shock proteins (HSP). Like the HSPs, the α-crystallin proteins can act as molecular chaperones (Horwitz, 1992). Members of the α-crystallin family were first discovered outside the lens being upregulated after damage to heart muscle (Bhat et al., 1991) and it is now believed that the α-crystallins can be cytoprotective (Kannan et al., 2012). Less is understood about the extralenticular function and expression of the β- and γ-crystallins. These two classes of proteins appear to be structurally similar and form a βγ-crystallin superfamily related to microbial spore coat proteins. Like αB-crystallin, they can be induced by physiological stress (Jaenicke and Slingsby, 2001). Members of the crystallin families of proteins are upregulated as a cellular response mechanism against stressors, such as osmotic

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stress, various types of injury, as well as bacterial infections in a number of different tissue types (Graw, 2009; Santana and Waiswo, 2011). The current data suggest that overexpression of crystallins, especially the α-crystallin family members, provides a protective mechanism for blocking apoptosis in neurons (Rao et al., 2008). In the retina the expression of crystallin proteins is altered following trauma and may play a protective role (Ahmed et al., 2004; Templeton et al., 2009; Vazquez-Chona et al., 2004).

Recently, our group has found what appeared to be a dramatic difference in expression of some crystallins in the retinas of different strains of mice (Templeton et al., 2009), consisting of high levels of crystallins in the C57BL/6 mouse and relatively low levels in the DBA/2J mouse. Further, following optic nerve crush these members of the crystallin family appear to respond in a coordinated manner, showing decreased expression in the C57BL/6 mouse retina and increased expression in the DBA/2J mouse retina. We hypothesized that these crystallin proteins might be regulated by a common molecular mechanism within the retina following injury. We use the BXD recombinant inbred (RI) strain set to examine the genetic regulation of the crystallins in the retina. This strain set is uniquely suited for this analysis since the two parental strains – C57BL/6 and DBA/2J – display a difference in crystallin expression (Templeton et al., 2009). In the present paper, we demonstrate the presence of a genetic network regulating the coordinated expression of a group of crystallin family members. This network contains a surprising combination of crystallin and non-crystallin genes, many of which are selectively or highly expressed in the lens, and may play a prominent role in the response of the retina and CNS to injury. We carry out a bioinformatic comparison of the promoter and 5′ regions of the co-regulated and control genes to identify functional and regulatory modulators and their gene interactions (Bloemendal and de Jong, 1991; Hejtmancik et al., 2001). The correlation in crystallin network expression across the BXD RI strains allows statistical analysis that identifies the Maf family and probably MafA as a putative transcription factor that potentially co-regulate this crystallin genes expression. The bioinformatic analysis was validated by chromatin immunoprecipitation (ChIP) in human lens epithelial cells (HLE) and rat retinal ganglion cells (RGC).

2. Materials and methods

2.1. Animals

All animals used were in compliance with institutional guidelines and with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center approved all protocols involving the use of mice. In the present study 35 mice were used to quantify protein and 9 were used for immunohistochemistry.

2.2. GeneNetwork databases

In the present study we defined the crystallin network and examined the levels of crystallin mRNA using three databases within the GeneNetwork website (genenetwork.org) and the Hamilton Eye Institute (HEI) Retina Database. Descriptions of the dataset can be found in the Information File attached to database on GeneNetwork.org.

2.3. Antibodies used

The antibodies directed against members of the crystallin family are: rabbit anti-γ-crystallin, rabbit anti-β-crystallin, rabbit anti-βA3/A1-crystallin, and rabbit anti-α-crystallin. All of these anti-crystallin antibodies were a gift from Dr. Sam Zigler. An additional rabbit anti-αB-crystallin was purchased from Abcam (ab13497, Abcam, Cambridge MA). Mouse anti-NeuN was purchased from Chemicon (Temecula, CA). The secondary antibodies were goat anti-rabbit antibody labeled with Alexa Fluor 488 (A11034, Invitrogen, Eugene OR), goat anti-mouse labeled with Alexa Fluor 568 (A11031, Invitrogen). TO-PRO-3 iodide was purchased from Invitrogen (T3605, Invitrogen, Eugene OR). The antibody used for immunoblots was goat anti-rabbit IgG peroxidase conjugated (Promega, Madison, WI).

2.4. Immunoblot analysis

For our immunoblot analysis mice were selected from a number of different BXD RI strains. The mice were deeply anesthetized with a mixture of 13 mg/kg of Rompum and 87 mg/kg of Ketalar and then killed by cervical dislocation. The retinas were removed and stored in sample buffer at −20 °C. Unless it is specifically stated, two retinas from each animal were pooled and placed in sample buffer (100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% SDS, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% 2-mercaptoethanol, and 10% glycerol) containing protease inhibitors (Roche) and immediately frozen. Samples were lysed by sonication on ice. The protein concentration of each sample was determined using the Bradford protein assay kit (Bio-Rad). Six standard BSA (0.25, 0.5, 0.75, 1, 1.5, and 2 mg/ml) were used to make a standard curve ($R^2 = 0.9$). The total protein concentrations of each sample were then measured. The concentration of each sample was then adjusted to 50 μg of protein to 100 μl of sample. The proteins (15 μg/lane) were separated on a 4–12% SDS-polyacrylamide gel and transferred to PVDF membrane (Immobilon-P; Millipore, Billerica, MA). After incubation with primary antibodies, the membranes were treated with anti-rabbit peroxidase-conjugated secondary (Promega, Nashville TN) for 2 h. Immunoreactive bands were detected by chemiluminescence (ECL western blotting reagents; Pierce, Rockford, IL). As loading control samples were also stained an antibody against β-actin (SC-47778, Santa Cruz Biotechnology, Santa Cruz, CA). Bands were quantified using a Kodak image analyzer and band intensities were analyzed by densitometry.

2.5. Immunohistochemistry

For immunohistochemical experiments mice were deeply anesthetized with a mixture of 13 mg/kg of Rompum and 87 mg/kg of Ketalar and perfused through the heart with saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.3). The retinas were placed in 4% paraformaldehyde overnight. The next day they were rinsed in phosphate buffer and then embedded in 4% agarose gel. The retinas were sectioned with a Vibratome at 100 μm thick. The sections were extensively rinsed in PBS, and then placed in blocking buffer containing 2% bovine serum albumin (BSA), 0.05% DMSO and 0.05% Tween-20 for 30 min. For immunohistochemistry, sections were incubated in primary antibodies (1:500) against γS-crystallin and αB-crystallin and incubated overnight at 4 °C. After rinsing the sections were incubated with secondary antibody conjugated to Alexa Fluor-488 (A21206, Invitrogen Molecular Probes) (1:1000) for 2 h at room temperature. The sections were then rinsed 3 times, flooded with Fluoromount-G, and covered with a coverslip. All images were photographed using on Nikon Eclipse TE2000-E confocal microscope at 40× and acquired by Nikon's EZ-C1 Software (Bronze Version, 3.91).