Involvement of Retinoic Acid/Retinoid Receptors in the Regulation of Murine αB-crystallin/Small Heat Shock Protein Gene Expression in the Lens*

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Crystallins are a diverse group of abundant soluble proteins that are responsible for the refractive properties of the transparent eye lens. We showed previously that Pax-6 can activate the αB-crystallin/small heat shock protein promoter via the lens-specific regulatory regions LSR1 (−147/−118) and LSR2 (−78/−46). Here we demonstrate that retinoic acid can induce the accumulation of αB-crystallin in N/N1003A lens cells and that retinoic acid receptor heterodimers (retinoic acid receptor/rexinoid X receptor; RAR/RXR) can transactivate LSR1 and LSR2 in cotransfection experiments. DNase I footprinting experiments demonstrated that purified RAR/RXR heterodimers will occupy sequences resembling retinoic acid response elements within LSR1 and LSR2. Electrophoretic mobility shift assays using antibodies indicated that LSR1 and LSR2 can interact with endogenous RAR/RXR complexes in extracts of cultured lens cells. Pax-6 and RAR/RXR together had an additive effect on the activation of αB-promoter in the transfected lens cells. Thus, the αB-crystallin gene is activated by Pax-6 and retinoic acid receptors, making these transcription factors examples of proteins that have critical roles in early development as well as in the expression of proteins characterizing terminal differentiation.

The refractive properties of the transparent eye lens depend on a diverse group of globular proteins called crystallins that comprise approximately 90% of the water-soluble proteins of this tissue (1, 2). Despite their specialized function in the lens, crystallins are surprisingly diverse and may differ among species. Moreover, crystallins often play more than one biological role, a situation called gene sharing (3), with many being related or identical to metabolic enzymes or stress proteins (4–6). These multifunctional crystallins are expressed very highly in the lens and to a lesser extent in other tissues, where they have nonrefractive roles.

The molecular basis for the specialized expression of crystallin genes has been investigated for some time (7). While no one cis-control element or transcription factor is solely responsible for the high lens expression of the crystallin genes, Pax-6 (8–11) and retinoic acid (RA)1 (12–14) appear to have prominent roles. This is consistent with the critical use of these transcription factors for eye and lens development (15–28).

We have been studying mouse αB-crystallin, a conserved small heat shock protein (29, 30) that is constitutively expressed highly in the lens and more moderately in many other tissues (31, 32). αB-crystallin is also induced by stress (33) and overexpressed in numerous diseases (34, 35). The differential constitutive expression of the murine αB-crystallin gene is developmentally and transcriptionally controlled (32, 36, 37). Transgenic mouse experiments have established that the sequences downstream of −164 are sufficient to direct lens-specific gene expression (38). This 5′-flanking sequence contains two lens-specific regulatory regions called LSR1 (−147/−118) and LSR2 (−78/−46). Pax-6 can interact with both LSR1 and LSR2 and activate the αB-crystallin promoter in transient transfection experiments (39). In the present study, we show by DNase I footprinting, antibody/electrophoretic mobility shift assay (EMSA), site-directed mutagenesis, and transient-co-transfection experiments that RAR/RXR heterodimers can interact with retinoic acid-responsive elements (RAREs) within LSR1 and LSR2 and can activate the αB-crystallin promoter in lens cells either alone or collaboratively with Pax-6.

EXPERIMENTAL PROCEDURES

Nucleic Acid Isolation—For transfection experiments, plasmid DNA was isolated and purified using the Qiagen plasmid kit according to the manufacturer’s instructions (Qiagen Inc., Chatsworth, CA).

Northern (RNA) Analysis—Total RNA was isolated from N/N1003A cells (40) treated with RA (Sigma) by using the RNA Isolation Kit (Stratagene, La Jolla, CA) and subsequently fractionated by electrophoresis through a 1.5% agarose-formaldehyde gel. The RNA was transferred to a Duralon membrane (Stratagene, La Jolla, CA) and hybridized to a 230-base pair HindIII–BamHI restriction fragment from exon 3 of the mouse αB-crystallin gene (32). The probe was labeled by using the Ready-To-Go Random Prime Labeling System (Amersham Pharmacia Biotech). Prehybridizations were performed at 60 °C for 30 min, and hybridizations were carried out at 60 °C for 90 min by using QuickHyb (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Membranes were washed and autoradiographed as described previously (41). Methylene blue staining was performed as earlier (42) to monitor the integrity of RNA, the relative amounts of RNA loaded on the gel, and the efficiency of transfer to Duralon membranes. Membranes were exposed for autoradiography on Kodak XAR5 film at ~80 °C with an intensifying screen for 12 h.

Nuclear Extracts, Oligonucleotides, and Antisera—Nuclear extracts (9) were prepared from αTN4–1 (43) and N/N1003A lens cells. Complementary oligodeoxynucleotides were synthesized (model 380A synthesizer; Applied Biosystems) and annealed at a 1:1 molar ratio as described previously (44). The oligodeoxynucleotides were labeled on one strand using T4 polynucleotide kinase, and electrophoretic mobility

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1 The abbreviations used are: RA, retinoic acid; EMSA, electrophoretic mobility shift assay; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid-responsive element; CAT, chloramphenicol acetyltransferase.

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shift assays (EMSA) were performed as described previously (44). Double-stranded oligodeoxynucleotides LSR1, LSR2, and short LSR2 containing sequences −136 to −109, −78 to −28, and −73 to −48, respectively, of the αB-crystallin promoter were used for EMSAs. Antibodies RAR/RXR monoclonal antibodies (45) were generous gifts from Drs. Pierre Chambon and Maria Gaub (Centre National de la Recherche Scientifique, Strasbourg, France). Polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (anti-RXRα, catalog number sc 831; anti-RARα, catalog number sc 551; anti-RARγ, catalog number sc 552; anti-RARγ, catalog number sc 773; and anti-RXRα, catalog number sc 774).

EMSA and DNase I Footprinting—A polymerase chain reaction–generated fragment corresponding to the −190 to +40 sequence of the αB-crystallin gene was used for footprinting experiments with purified mouse RAR/RXR receptors. DNA and protein were incubated and treated with DNase I as described previously (44). The RAR/RXR proteins were kindly provided by Drs. Keiko Ozato and Jorge Blanco (NICHD, National Institutes of Health, Bethesda, MD). RARγ was obtained from Santa Cruz Biotechnology. End-labeling, EMSA, and DNase I footprinting were performed as described earlier (44).

Western (Protein) Analysis—Nuclear extracts prepared from αTN4–1 and N/N1003A cells were fractionated by electrophoresis in a Tris-glycine polyacrylamide gel; the separated proteins were transferred to nitrocellulose membranes using a Trans-Blot (Bio-Rad). Immunoblotting was performed according to the manufacturer’s instructions (Vector Labs, Burlingame, CA).

Site-directed Mutagenesis—Plasmids containing mutations generated previously (38) within the −164/+4 EcoRI/PstI fragment of the mouse αB-crystallin gene, cloned in pR30A (36), were used for transient transfection experiments and EMSAs. In brief, site-specific mutations (Mu-9762 and Mu-9763) (38) were introduced by using an oligodeoxynucleotide-directed mutagenesis kit (Sculptor in vitro mutagenesis kit, Amersham Pharmacia Biotech). Mutated oligodeoxynucleotides contained the substitution sequence TCTAGA (Eco44I fragment of the B-crystallin gene promoter) and 20 bases on each side complementary to the B-crystallin promoter sequence. The resulting mutated restriction fragments were subcloned into pR30A at the unique BamHI site (36). All constructs were confirmed by sequencing the ligated junctions and mutated regions.

Cell Culture, Transient Transfections, and CAT Assays—Mouse COP-8 fibroblasts (46) and N/N1003A lens cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum and 50 µg/ml of gentamicin in 10% CO2. The cells were propagated on 60-mm diameter plastic dishes. 10 µg of wild-type αB-promoter-cat plasmids (p65–7 and p11–3) (36) or mutated test plasmids (Mu-9762 and Mu-9763) (38); increasing amounts (0.25–1 µg) of pSV40RARB and pRSV4RXRα (gifts from Drs. Keiko Ozato and Jorge Blanco), which express the wild-type RARβ and RXRα, respectively (47), and 2 µg of internal control pCH110, which expresses β-galactosidase (Amersham Pharmacia Biotech), were cotransfected for 6 h by the calcium phosphate method as described previously (44). Cells were treated with 100 µl of 0.1 ng/ml of RA in the morning following transfection for 1 h. The cells were harvested, and extracts were prepared 48 h after transfection. CAT activities were determined by the biphasic assay (48), and β-galactosidase activities were determined as described previously (44). The transfection data represent the means of three separate experiments, with each experiment being conducted in duplicate.

RESULTS

Northern Blot Hybridization of αB-crystallin mRNA—In order to test whether retinoid signaling can induce endogenous αB-crystallin gene expression in lens cells, Northern blot hybridizations were performed with total RNA isolated from N/N1003A cells treated with increasing concentrations of RA (Fig. 1). The intensity of hybridization of the labeled probe to αB-crystallin mRNA was approximately 3 times greater in the cells treated with higher concentrations of RA (Fig. 1, lanes 1–3). Control tests showed no increase in glyceraldehyde-3-phosphate dehydrogenase and α-actin mRNAs after treatment with RA (data not shown).

Western Blot Analysis for RAR/RXR Receptors—Nuclear extracts from N/N1003A and αTN4–1 lens cells were used to test for the presence of RAR/RXR receptors. Western blot analysis using anti-RAR and anti-RXR antibodies showed that both αTN4–1 and N/N1003A cells express RAR (Fig. 2, lanes 3 and 4) and RXR receptors (Fig. 2B, lanes 3 and 4), consistent with the induction of αB-crystallin gene expression by RA. Purified RXRα (Fig. 2A, lane 2) and RXRβ (Fig. 2B, lane 1) were used as positive controls in these tests.

DNase I Footprinting with Lens Nuclear Extract and RAR/RXR—We next examined the possibility that heterodimers of retinoic acid receptors can bind to the lens-specific sites LSR1 and LSR2. Three retinoic acid receptor heterodimers (RARβ/RXRγ, RIXRβ/RARγ, and RARβ/RXRβ) were tested for the ability to protect the −190/+40 fragment of the αB-crystallin gene from digestion with DNase. Fig. 3 shows that three regions were protected by each of the heterodimers tested, with the weakest footprint generated by RXRβ/RARγ. The protected regions comprised LSR1 (−132–110), LSR2 (−73–54), and a region between LSR1 and LSR2 (−106/−87). The LSR1 and LSR2 sequences was footprinted on both DNA strands; however, the intervening region (−106/−87) was not footprinted on the upper (sense) strand (data not shown).

The footprinted sequences and the surrounding nucleotides are shown in Fig. 4A. Regions that resemble the consensus binding sequence for RAR/RXR (12) are designated RARE in Fig. 4B; the nucleotides represented by the larger uppercase
Functional Cotransfection Tests with RARβ/RXRβ and Pax-6 cDNA Expression Plasmids—To test whether RARβ/RXRβ receptors can activate the αB-crystallin promoter, transient cotransfection experiments were performed using cDNA expression plasmids in N/N1003A lens cells. Vector alone (pRSV) did not activate the −164/+44 αB-crystallin promoter fused to the cat gene (p65−7) in cotransfected N/N1003A cells (data not shown). p65−7 contains LSR1 and LSR2. By contrast, cotransfection with a mixture of pSV40RARα and pRSVXRβ caused a 5−6-fold RA-dependent stimulation of CAT activity in the cells transfected with p65−7 (Fig. 7A). Cotransfection with either pSV40RARβ or pRSVXRβ alone, however, did not stimulate the reporter gene expression to a significant level (data not shown). pSV40RARβ and pRSVXRβ stimulated CAT expression approximately 3-fold in cotransfection experiments using p11−3, which contains LSR2 but lacks LSR1 (Fig. 7A). The absolute amount of CAT activity produced from p11−3 was at least 3-fold lower than that resulting from p65−7 (data not shown). Site-specific mutations Mu-9760 and Mu-9761 were compared with the wild-type construct (p65−7) for their ability to direct expression of the cat gene in N/N1003A cells cotransfected with pSV40RARα and pRSVXRβ. The αB-crystallin promoters containing the Mu-9762 and Mu-9763 mutations were only about half as responsive as the wild type promoter in p65−7 to
stimulation by pSV40RARβ and pRSVRXRβ in the cotransfected cells (Fig. 7B).

Finally, since both Pax-6 (39) and RARβ/RXRβ (above) can stimulate αB-crystallin promoter activity, we tested whether these transcription factors have an additive or synergistic effect in cotransfection experiments. First, we confirmed that the Pax-6 expression plasmid, pKW10-Pax-6 (49), can stimulate CAT expression in N/N1003A lens cells transfected with p65–7, since our earlier experiments showing a maximum 5-fold increase in αB-crystallin promoter activity were performed in the COP-8 fibroblast cell line (39). The data in Fig. 8A show that pKW10-Pax-6 has a similar effect on p65–7 activity in the cotransfected N/N1003A cells as previously in COP-8 cells, even with respect to the decrease at higher concentrations of pKW10-Pax-6. Similar results were obtained with pKW10-Pax-6 and p11–3, which contains only LSR2 (data not shown). Cotransfection experiments using pKW10-Pax-6, pSV40RARβ, and pRSVRXRβ in conjunction with p11–3 (Fig. 8B) or p65–7 (Fig. 8C) showed that Pax-6 and RARβ/RXRβ have an additive stimulatory effect on αB-crystallin promoter activity. The additive stimulation was approximately twice as great with p65–7 as with p11–3. These data suggest that LSR1 and LSR2 are regulatory regions that utilize both Pax-6 and RAR/RXR for αB-crystallin promoter activity.

**DISCUSSION**

We have shown previously in transgenic mice using reporter transgenes that the high lens and lower nonlens expression of the mouse αB-crystallin gene are developmentally controlled at the transcriptional level (36, 37). Lens-specific expression has been localized to LSR1 (–147/–118) and LSR2 (–78/–46), with the minimal lens-specific αB-crystallin promoter fragment identified being the –115/+44 sequence (38, 39). The combined presence of LSR1 and LSR2 in the –164/+44 promoter fragment is approximately 30 times more active in the lens than is LSR2 alone in the minimal promoter fragment in transgenic mice (39). Promoter activity is augmented approximately 7-fold in the transgenic mouse lens when the –426/+44 promoter fragment is used, which includes an enhancer at positions –426/–259 (36, 38). Thus, high lens activity of the mouse αB-crystallin gene depends on coupling multiple cis-control elements and their cognate transcription factors.

We have shown earlier and confirm here that Pax-6 can activate the αB-crystallin promoter via both LSR1 and LSR2 in transient transfection experiments (39). Pax-6 also contributes to the lens expression of the mouse (9) and chicken (8) αA-, the chicken δ1- (10), and the guinea pig ε- (11) crystallin genes (7). In recent transgenic mouse experiments, a mutant TATA-like sequence associated with LSR2 preferentially reduced αB-crystallin promoter activity in the lens in a Pax-6-independent fashion, indicating that, as expected, multiple factors contribute to the specialized activity of the αB-crystallin promoter in the lens (50). The present investigation shows that retinoic acid receptors, especially RARβ/RXRβ heterodimers, can also bind LSR1 and LSR2 and activate the αB-crystallin promoter. The

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**FIG. 4.** A, summary of DNase I footprinting for lower (antisense) strand. Footprints for RAR/RXR are shown as open boxes. B, alignment of RAR/RXR recognition sequences in mouse αB-crystallin promoter. Large uppercase letters indicate matching of the nucleotides with the consensus binding site for RAR/RXR.
activation of the αB-crystallin promoter by the simultaneous presence of RARβ/RXRβ and Pax-6 is additive rather than synergistic and leaves unresolved whether or not these factors physically interact with each other or through co-factors. Indeed, the mechanism of gene activation by retinoid receptors has not been established and involves chromatin alterations as well as direct interactions with DNA sequences (51). It remains to be determined if retinoic acid receptors play a role in the nonlens or stress-induction of the αB-crystallin/small heat shock protein (35).

Retinoic acid receptors are members of the superfamily of nuclear factors (thyroid hormone, steroid hormone, and vitamin D3 receptors) and are involved in a wide array of developmental processes (52–56). The importance of retinoid signaling for eye development in mice has been established by application of exogenous RA (57) and by deleting various combinations of RAR and RXR genes (18, 19, 58, 59). The existence of retinoic acid receptors in cultured lens cells was shown in our Western blots in the present experiments. A role of retinoid signaling for lens differentiation is implied by the generation of abnormal lens phenotypes by ectopic expression of cellular RA-binding protein 1 (15) and RAR (60) and by the expression of reporter genes driven by RAREs of the RARβ gene in the presumptive (61) and developing (20) lens of transgenic mice. It has also
been demonstrated that a minimal promoter-lacZ reporter gene fused to the RARE from the human RARβ-2 gene is expressed in the zebrafish as early as embryonic day 9.5 in specific embryonic regions including the optic cup (62). Thus, retinoic acid receptors and Pax-6 are both examples of general factors that play essential roles in the early development of the lens as well as in the regulated expression of crystallin genes, which encode the major proteins of the terminally differentiated lens (1). This is consistent with the idea that one of the selective mechanisms used for recruiting the multifunctional crystallins is their responsiveness to transcription factors required for the development and maintenance of the transparent lens (5, 6, 63).

So far our data show only that RXRβ and at least one of the RARs (α, β, or γ) are present in the N/N1003A and αTN4-1 lens cells. With respect to the intact lens, a broad complex forms with the LSR2 oligodeoxynucleotide and lens nuclear extract; however, this complex was unaffected by the addition of the set of RAR and RXR antibodies used in the experiments with the cultured cells (data not shown). Because of the overlap between the Pax-6 and RAR/RXR binding sites, it is possible that Pax-6, RAR, or RXR, and other factors present in the lens nuclei bind simultaneously at LSR2 and leave the antibody-interactive sites for the retinoic acid receptors unavailable. In any case, further experiments are necessary to establish unequivocally which retinoic acid receptors may be involved in the activation of the αB-crystallin gene in the cultured lens cells and in the intact lens.

The present results add to previous experiments indicating that RA and its receptors play a critical role in the regulation of crystallin genes in the lens. RA has been shown to activate the d1-crystallin gene in stably transformed mouse teratocarcinoma stem cells (64) and in cultured lens epithelial cells from newly hatched chickens (17). Recent cotransfection experiments using reporter genes in recombinant plasmids have provided more direct evidence implicating retinoic acid receptors in the control of the chicken δ1-crystallin gene (14). Unlike the
δ1-crystallin promoter/enhancer, the δ2-crystallin promoter/enhancer is not stimulated by RARβ in the cotransfected primary lens epithelial cells. This differential responsiveness is particularly interesting, since δ2-crystallin, an active arginino-succinate lyase, is present at a relatively low concentration in the chicken lens, while enzymatically inactive δ1-crystallin is the major δ-crystallin in the lens (3, 65).

Extensive experiments have demonstrated that the mouse γF-crystallin gene is controlled by retinooid signaling. It was first shown that RAR/RXR heterodimers bind to a novel everted RARE (called γF-HRE) consisting of two half-sites separated by eight base pairs in the 5'-flanking sequence (66). The regulation of the γF-crystallin gene by retinoic acid receptors appears very complex, inasmuch as γF-HRE is activated by T3/RXR as well as RAR/RXR, yet is repressed by T3/RAR (13). There is also an RAR-related orphan receptor, RORα, that is expressed in the mouse lens and binds as a monomer to the γF-HRE 3'-half site and spacer sequences (66). RORα stimulates γF-crystallin promoter activity in transfected primary chicken lens epithelial cells. Moreover, RORα occupancy and promoter activation are blocked by competing RAR/RXR heterodimers in the absence of RA; the blockage of RORα activation of γF-HRE by RARα is dose-dependent and similar to the repression of the T3 response from γF-HRE reporter plasmids. The novelty of γF-HRE (12) and the involvement of RORα, which does not compete for binding to β-RARE or TRE (66), raises the possibility that the stimulation of the γF- and δ-crystallin promoters operates by different pathways.

Many invertebrates have complex eyes with cellular lenses containing abundant crystallins (67). Virtually nothing is known about the developmental pathways controlling the development of these lens-containing invertebrate eyes or about the regulatory mechanisms used for expressing their crystallin genes. Recent experiments have raised the possibility that retinoid signaling may extend to crystallin gene expression in invertebrates. Two novel lens crystallin genes (J1A- and J1B-crystallin) cloned from the cubomedusan jellyfish (Tripedalia cystophora) (68) have RARE half-sites in their promoter regions, and these sequences bind a cloned RXR homologue derived from the same species.2 If a causal connection can be made between retinoic acid receptors and J-crystallin gene expression, it would provide strong evidence that retinoid signaling is a conserved pathway for crystallin gene expression throughout the animal kingdom.

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