Biol. Chem., Vol. 378, pp. 1331–1348, November 1997 ·

Review

The Crystallins: Genes, Proteins and Diseases

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The crystallins were discovered as the structural proteins of the vertebrate eye lens in the last century by C.T. Mörner (Z. Physiol. Chem. *18*, 1893, 61–106). Since that time the mammalian crystallins referred to as α-, β-, and γ-crystallins have been characterized with respect to their genetic organization, the regulation of their expression pattern and their participation in several diseases. Moreover, some crystallins have also been discovered outside the eye. Evolutionary analysis has demonstrated the relationship of crystallins to proteins involved in protection against stress.

The α-crystallins are considered to be molecular chaperones and members of the small heat shock protein family; they have autokinase activity and are involved in the γ-crystallin gene activation. The α-crystallins are associated with a broad variety of neurological disorders.

The β/γ-crystallin superfamily is characterized by four greek key motifs. The various N- and C-terminal extensions of the β/γ-crystallins are mainly responsible for their distinct biophysical and biochemical properties. Modifications in the β/γ-crystallins or mutations in their genes lead to opacification of the eye lens (cataract).

Other proteins found to be expressed at relatively high levels in the lens are characterized by their strong relationship to well-known enzymes. They are referred to as enzyme-crystallins, and as one example, the ξ-crystallin will be discussed. It has evolved from a quinone oxidoreductase using a lens-specific promoter, and a mutation in ξ-crystallin is involved in cataract formation.

Key words: Cataract / Crystallin / Evolution / Gene Expression / Inherited Disease / Lens.

Introduction

The vertebrate eye lens is a unique organ: transparent and flexible, derived from only one cell type, and containing a high amount of protein. These proteins represent about 30–35% of the entire mass of the lens – the water content, which is usually approximately 95% in a cell, is reduced in the lens to 65–70%. The high density of packing needs

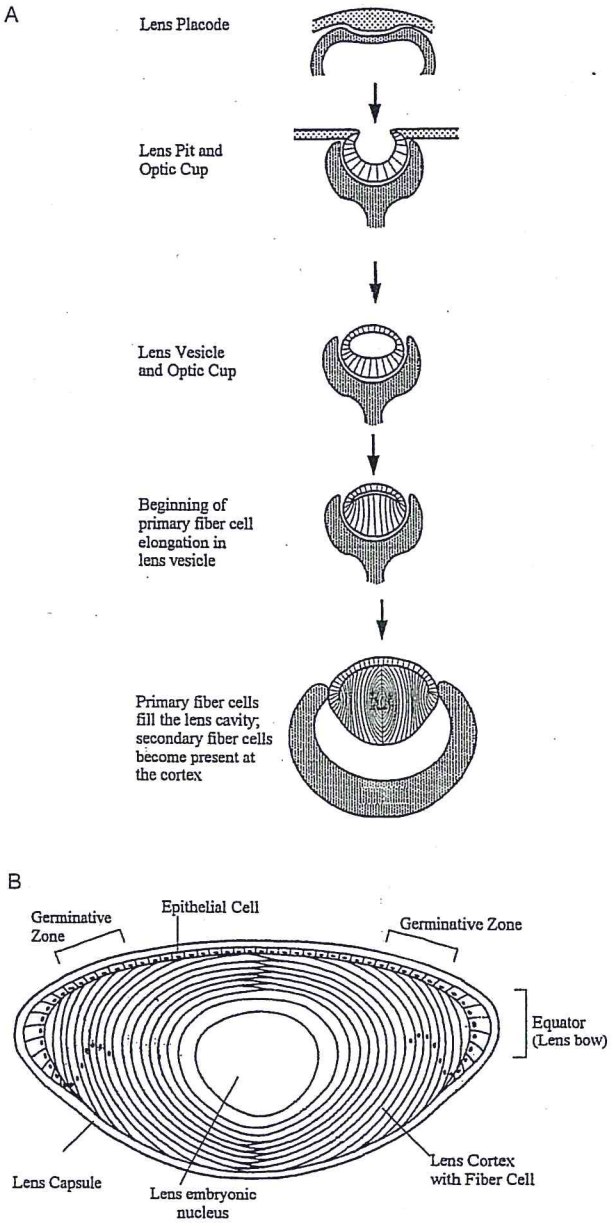

Fig. 1 Survey of Lens Development and Differentiation.
(A) The vertebrate lens develops from the lens placode within the surface head ectoderm by invagination forming a lens pit and later the lens vesicle. The cavity of the lens vesicle is filled from posterior by the primary fiber cells, later forming the lens nucleus. Epithelial cells remain at the anterior part.
(B) The juvenile and adult lens is separated from surrounding fluids by a capsule. The anterior epithelial cells divide in the ring-like germinative zone (appearing twice in the cross-section). In the equatorial zone (lens bow) they begin to differentiate. Secondary fiber cells become present in the lens cortex. Moreover, the lens is characterized by the ongoing breakdown of the fiber cell nuclei in the deeper cortex.

highly specialized proteins. When in 1893 Mörner analyzed the bovine lens proteins, he referred to them as crystallins because of their abundance in the crystallin lens. The classification as α-, β- and γ-crystallins followed decreasing molecular weight and increasing isoelectric point of the native proteins, and in general it has been valid up to now. However, the view today leads not only to a diversification of the original protein families, but also to the discovery of several cellular functions of the crystallins in addition to their properties as structural proteins.

To understand the various aspects of crystallin function it is necessary to summarize briefly the development and differentiation steps of the eye lens. Lens development starts by the invagination of the lens pit at the places of the lens placode at both sides of the prospective forebrain. In these areas ectoderm from the diencephalon has close contact to the surface ectoderm. This initial process takes place in the mouse embryo at day 9.5 of embryonic development (E9.5). The lens placode indents to form the lens pit and subsequently the lens vesicle at E11.5. From the posterior side the primary lens fiber cells grow into the lumen and fill the vesicle at E13. In man, this process takes place between the fourth and sixth weeks of gestation. From that time on, a lifelong process of formation of secondary fiber cells is initiated. At the germinative zone of the anterior lens epithelium the cells divide and move to the lens equator, where they elongate to the anterior and posterior pole of the lens surrounding the earlier fiber cells. Since this process takes place throughout life, sections of the lens look like annual rings of a tree with the outermost, superficial fiber cells being the youngest. During the process of the terminal differentiation of the lens fiber cells, all cell organelles are degraded leading finally to cells without nuclei and mitochondria in the center of the lens. The developmental process is completed for the mouse lens two weeks after birth, when the eye lids are separated. In man, this process is completed during the second trimester and is schematically summarized in Figure 1. The ongoing process of nuclear breakdown in the permanently differentiating secondary fiber cells has some similarities with the initial steps in processes referred to as apoptosis in other tissues. It is dependent on a variety of signals from different sources. These influences are summarized elsewhere (Cvekl and Piatigorsky, 1996; Graw, 1996) and will not be discussed here.

α-Crystallins

Gene Structure and Evolution

The α-crystallin complexes are mainly composed of two related proteins, αA- and αB-crystallin. They are encoded by two genes, *Cryaa* and *Cryab*, which are located on different chromosomes. The basic knowledge about α-crystallins is summarized in Table 1.

Cryaa and *Cryab* have been determined in a variety of species (e.g. mouse, man, hamster, rat, chicken, rabbit); both genes contain three exons of similar size. In rodents (mouse, rat, hamster, rabbit) an alternative splice product can be observed in 10–20% of the αA-crystallin. From intron A an additional 69 bp are included in the mature mRNA leading to a protein 23 amino acids longer than the usual αA-crystallin; it is referred to as αA^{ins}-crystallin. It is still not known whether there is any function for this particular splice product, or if it might be only a transient product of evolution in rodents.

Gene Expression and Regulation

The expression of the two *Crya*-genes is not uniform, even if both are expressed at very high levels in the lens. The αA-crystallin can be considered as a lens-specific protein, since only trace amounts can be found outside of the lens in the spleen (Kato *et al.*, 1991; Krausz *et al.*, 1996). During embryogenesis, *Cryaa* expression is observed in the rat lens pit (van Leen, 1987) and in the mouse lens cup at E10 to E10.5 (Robinson and Overbeek, 1996). It is present in the posterior half of the lens vesicle (Tréton *et al.*, 1991), and later on, αA-crystallin becomes very abundant in lens fiber cells (Robinson and Overbeek, 1996).

On the other hand, *Cryab* is expressed ubiquitously. In rat lenses, αB-crystallin can first be detected at the end of embryonic development (Aarts *et al.*, 1989), however, in the mouse, it is present at E9.5 and later on is found preferentially in the epithelial cells (Robinson and Overbeek, 1996). Transcripts can also be found in remarkable amounts in brain, heart, skeletal muscle, lung, thymus and kidney and a variety of cell lines (Krausz *et al.*, 1996). During embryogenesis, expression of *Cryab* mRNA was detected by *in situ* hybridization in the primitive heart of the mouse at E8.5 and in the myotome of the somites at E10.5, supporting the hypothesis that functions of αB-crystallin

Table 1 The α-Crystallins

Gene	Chromosome		Protein	Organs of expression	Function
	Mouse*	Human			
Cryaa	17 (17.4)	21	αA-crystallin; 20 kDa αA^{ins}-crystallin; 25 kDa	lens, (spleen)	Structural protein Chaperone Autokinase Gene activator
Cryab	9 (29.0)	11q12–q23	αB-crystallin; 22 kDa	lens, heart, brain, muscle, kidney	structural protein heat shock protein

* cM position according to the Mouse Genome Database (1997) in brackets.

might be coupled to the activation of genetic programs responsible for myogenic differentiation and cardiac morphogenesis (Benjamin *et al.*, 1997).

To address the different expression patterns of αA- and αB-crystallin, the promoters of the two genes have been compared recently (Cvekl and Piatigorsky, 1996). It turned out that for both *Crya* genes the presence of *Pax6* is necessary to initiate their expression to the lens; *Pax6* is widely accepted as a master control gene of eye development (Halder *et al.*, 1995). The *Cryaa* promoter from −364 to +45 was demonstrated to direct different reporter genes in transgenic mice exclusively to the lens (Overbeek *et al.*, 1985). This region has a complex array of positive and negative elements including an αA-crystallin binding protein, Pax6, a cAMP-responsive element and two further promoter elements referred to as PE1B and PE2. Additionally, the −7/+5 region of the *Cryaa* promoter exhibits sequence similarity with the consensus upstream stimulating factor (USF) binding site. Sax *et al.* (1997) demonstrated that USF can bind to this particular sequence, and their data support the possibility that USF plays a role in promoter activity of this gene.

For *Cryab*, a 4 kb promoter fragment fused to the *lacZ* reporter gene is specifically expressed in the lens (Haynes *et al.*, 1996). Only 1800 bp of the promoter are needed for lens preferred activity of a reporter gene if the fragment is stably integrated into the chromatin (Sax *et al.*, 1996). Further dissection of the promoter revealed that elements between −147 and −118, as well as between −115 and −69, interact with Pax6 and additional nuclear proteins as yet unidentified (Gopal-Srivastava *et al.*, 1996). These two sites seem to be responsible for the lens-specific expression.

In tissues outside the lens, *Cryab* is controlled by tissue-specific activators. In skeletal muscles, its expression is regulated by the MyoD family of basic helix-loop-helix transcription factors during myogenesis. The strong muscle enhancer sequences include a canonical E-box, an AP-2 site and a repeat sequence known to be important for HSF binding. A heart-specific element is recognized, which is not used in the lens or skeletal muscle. This responsible element contains the HSE and a reverse CArG-box. From footprint experiments it might be concluded that the E-box is also necessary for the expression in heart cells (Gopal-Srivastava *et al.*, 1995). Interestingly, in lung and brain a far upstream transcription initiation site localized to −474 is used (Frederikse *et al.*, 1994). Recently, the presence of alternative transcriptional initiation and of polyadenylation signals in the *Cryab* gene was reported in different chicken tissues leading to at least four distinct types of cDNA. The two principal forms found in lens and embryonic tissues possess a short 5′-UTR and differ in the length of the 3′-UTR. Forms with longer 5′-UTR are present in testis, muscle and heart (Macip *et al.*, 1997).

After heat or arsenite stress, the HSE is used by the heat shock transcriptional factor in C6 rat glioma cells similar to the transcription of *hsp27*; the stress response can even be enhanced by cyclooxygenase inhibitors. These results indicate that the response to stress of αB-crystallin is coupled with the metabolic activity of phorbolester and okadaic acid (Ito *et al.*, 1995) as well as of the arachidonic acid cascade (Ito *et al.*, 1996). Further studies revealed that prostaglandins prolong the arsenite-induced effect (Ito *et al.*, 1997).

Proteins and Function

The α-crystallins represent the major class of water soluble proteins in the lens (about 30%). The native α-crystallin in the eye lens is a large complex composed of the two subunits αA- and αB-crystallin and a molecular mass of approximately 800–1 000 kDa; the isolated subunits have molecular masses of 20 and 22 kDa (αA- and αB-crystallin, resp.), and the isoelectric points of the native proteins were reported ranging from 4.5 to 5.0 (Rink *et al.*, 1982; de Jong, 1982). However, the cDNA sequence of mouse and rabbit αB-crystallin predicts an isoelectric point of 7.8. The α-crystallins have been considered to be structural proteins and the object of a variety of post-translational modifications (truncation, glycosylation, glycation, carbamylation and acetylation; for review see Groenen *et al.*, 1994).

One of the most important modifications is phosphorylation. Past studies have demonstrated that the major site of *in vivo* phosphorylation is Ser^{122} in αA-crystallin and Ser^{45} in αB-crystallin. Although it has been well known for several years that the Ser^{122} in αA-crystallin is phosphorylated in a cAMP-dependent manner (Spector *et al.*, 1985), the function of this phosphorylation event remains to be elucidated. This phosphorylated form of αA-crystallin could be detected in human lenses only from adolescent, adult and senile donors, but not in infants, suggesting a developmental regulation of this particular kind of modification (Takemoto, 1996a). Moreover, α-crystallin, particularly αA-crystallin, has an autokinase activity (Kantorow and Piatigorsky, 1994). The phosphorylation site of the autokinase activity is different from Ser^{122}, and most likely it is one of the three Ser residues in the fragment 131–145. This autophosphorylation is stimulated 10-fold by the deoxycholate-mediated conversion of α-crystallin oligomers to tetramers (Kantorow *et al.*, 1995). The function of the autophosphorylation mechanism of αA-crystallin is still unknown.

Because of the high molecular weight of the native protein, several models for the structural organization have been proposed. Structural predictions were mainly done to explain the highly densely packed organization of α-crystallins in the lens. Based upon the gene structure, Wistow (1985) thought the overall structure of α-crystallin to consist of a globular N-terminal domain of two symmetry-related motifs and a somewhat longer C-terminal domain (also consisting of two domains) with an exposed C-terminal tail. The two globular domains, which are built up by two exons, are fused by a short connecting peptide. This short connecting peptide is extended in the rodent $αA^{ins}$-crystallin.

Bindels *et al.* (1979) first proposed a 'three-layer model' for the quarternary structure of the complex. According to their position in the complex, three classes of αA-crystallin subunits of different degree of surface exposure exist. Based upon data from Merck *et al.* (1992), Wistow (1993a) suggested a tetramer to be the fundamental building block for large aggregates. Among several possible forms, the dodecahedron is discussed to be one of the stable forms of α-crystallin. The quarternary model suggested by Carver *et al.* (1994) implies a hole of approximately 55 Å in the center of the aggregate to ensure equivalence for all subunits. The proposed structure consists of two layers of approximately 20 subunits with each layer being an annulus. Each subunit of α-crystallin is arranged such that its hydrophobic N-terminal domain is oriented away from the solvent and somewhat protected by the larger hydrophilic C-terminal domain. The interior of this hole is lined with hydrophobic residues from the N-terminal domain to provide a large hydrophobic area. The flexible C-terminal extensions (eight to ten amino acids) that protrude from the C-terminal domain cover the hole at both ends. The model is based on the knowledge that the hydrophilic C-terminal domain of each subunit is exposed to the surface and imparts aggregate solubility; the nonpolar regions of the molecule (i.e. the hydrophobic N-terminus) are segregated from the solvent. This leads to a significant reduction in free energy, with a strongly positive entropy; i.e. entropy is the driving force for subunit aggregation. Once formed the complex is stabilized by a combination of the entropic effects and interactions of the hydrophilic C-terminal domains (Augusteyn and Koretz, 1987).

However, a recent paper from Stevens *et al.* (1996) demonstrated with electron microscopic data the existence of sheet-like structures of α-crystallin, extracted from cross-linked lens extracts. Additionally, an 'open' dynamic micellar structure composed of αA-crystallin subunits has been proposed to explain that differences in ionic strength during maturation of fiber cells lead to alteration in α-crystallin interactive properties by inducing a decrease in effective charge and an increase in protein-protein interaction (Groth-Vasselli *et al.*, 1995; Singh *et al.*, 1996). This model is also compatible (Farnsworth *et al.*, 1997) with new data that the hydrophobic region of αA-crystallin at amino acids 32–37 has an increased deuterium exchange with increasing temperature. This enhanced exchange is attributed to an increase in hydrophobic surface exposure, which might be responsible for further interactions of αA-crystallin with other proteins (Smith *et al.*, 1996). The ongoing debate on the quarternary structure of α-crystallin might also point to divergent structures of the large complex based on different composition subunits.

In 1982, based upon sequence comparison, Ingolia and Craig identified the homology between the small heat shock proteins (sHSPs) from *Drosophila* and the α-crystallins. In a region containing 76 amino acids, 39 amino acids are conserved in α-crystallin and the sHSPs, which leads to the definition of a common motif of about 80 amino acids, the so-called α-crystallin domain (Caspers *et al.*, 1995). This motif can also be found in very divergent proteins, including surface antigens in parasitic eukaryotes and bacteria. It was used to construct a phylogenetic tree indicating a monophyletic origin of the animal sequences. The αA- and αB-crystallins form a clade together with the rat and human p20 and hsp27. αA-crystallin has been overexpressed both by stable transfection of HeLa cells and by transient transfection of NIH 3T3 cells. In both experimental systems αA-crystallin overexpression resulted in an increased cellular thermoresistance as judged by different clonal survival assays, demonstrating that αA-crystallin is a functional member of the sHSP family (van den Ijssel *et al.*, 1994). Moreover, the intron/exon structure in the mouse *sHSP25*, human *sHSP27* and *Crya* are conserved. The homology of DNA and amino acid sequences between α-crystallins and sHSPs is also reflected by the presence of HSE in the *Cryab* promoter.

The accumulation of αB-crystallin outside of the lens (heart, brain, muscle, kidney) has been correlated with cells of high oxidative capacity. A recent report by Neufer and Benjamin (1996) demonstrated a 20-fold increase of *Cryab* transcription in skeletal muscle after continous low frequency motor nerve stimulation. In ocular trabecular meshwork different responses of *Cryab* expression were observed against heat-shock and oxidative stress. A transient change in mRNA mobility was observed only after heat-shock (Tamm *et al.*, 1996). In glial cells, Kegel *et al.* (1996) demonstrated the selective induction of αB-crystallin by osmotic stress; a recombinant expression of *Cryab* in these cells makes them resistent against acute hypertonic stress. In contrast to previously mentioned systems, *in vitro* cultures of murine L929 cells do not express αB-crystallin upon exposure to thermal stress. These cells appear to have rendered the *Cryab* gene locus inactive through methylation, thus providing a unique system by which to study the function of transfected αB-crystallin (Blackburn *et al.*, 1997).

A novel area of the common mode of action among sHSPs and α-crystallins was opened up by the observations of Mehlen *et al.* (1996b). The expression of αB-crystallin, as well as human or *Drosophila* sHSP27, confers resistance to apoptotic cell death induced by staurosporine, a protein kinase C inhibitor. The authors concluded that small stress proteins including αB-crystallin might be regulators of apoptotic pathways. The same group (Mehlen *et al.*, 1996a) also demonstrated that αB-crystallin and sHSPs protect murine L929 cells against TNFα-mediated cell death by inhibiting the mechanisms downstream of TNFα (like NF-κB activation, lipid peroxidation, and protein oxidation). On the other hand, the different sHSPs (including αB-crystallin) raised the total glutathione level, which is considered to be essential for the protective mechanism against TNFα-mediated cell death and probably some types of oxidative stress.

One of the most exciting finding concerns the function of α-crystallin as a molecular chaperone as outlined first in detail by Horwitz (1992). His original finding demonstrated

that α-crystallin prevents thermal aggregation of several enzymes and β/γ-crystallins. In contrast to the *Escherichia coli* chaperone GroE, the α-crystallin is a kind of suicide chaperone. It binds denatured protein and keeps it in solution. The high molecular weight fraction of the α-crystallins, α_{HM}-crystallin (50–300 kDa), is discussed as a biochemical correlate to a waste box (Srivastava *et al.*, 1996). Blakytny and Harding (1996) extended the chaperone function of α-crystallin to the prevention of the fructose-induced inactivation of enzymes in the lens, since fructose was shown to be the most rapidly acting sugar in inactivating enzymes. The presence of divalent cations like Ca^{2+} and Mg^{2+} (Koretz *et al.*, 1997), as well as the age-dependent conversion of α-crystallin into the α_{HM}-crystallin protein (Carver *et al.*, 1996), reduces the efficiency of the chaperone function of α-crystallins.

Chaperone activity is essential for the lens because degradation and extrusion of defective proteins is not possible as it is in other tissues. Moreover, the lens is exposed to a variety of damaging agents, in particular light of various wavelength, which leads to peroxidative effects on quite a number of lens proteins. This point of view is supported by the finding of Das and Surewicz (1995) demonstrating α-crystallin has a substrate specificity different from other chaperones and recognizes specific non-native intermediates formed during denaturation only, with no affinity for intermediates on the refolding pathway.

The chaperone activity of α-crystallin was first localized to amino acid residues 158–173 of the C-terminal region of αA-crystallin (Takemoto, 1994), but recently, reduced chaperone-like activity was demonstrated in the alternative splicing product αA^{ins}-crystallin (Smulders *et al.*, 1995a), as well as in αA-crystallin mutations ($Asp^{69} \rightarrow Ser$; Smulders *et al.*, 1995b) and the introduction of a hydrophobic tryptophan (Smulders *et al.*, 1996).

Recently, van Boekel *et al.* (1996) investigated the influence of some post-translational modifications. Glycation and oxidative modification decreased the chaperone-like activity of α-crystallins; homopolymers of αA-crystallin had a higher protecting capacity compared with those of αB-crystallin. The *in vivo* phosphorylated forms revealed a somewhat better protecting ability than the respective non-phosphorylated forms. Besides prevention of thermal aggregation, Borkman *et al.* (1996) presented some experimental evidence that the chaperone activity of α-crystallin inhibits UV-induced protein aggregation.

The third important aspect of α-crystallin concerns its participation in the intracellular architecture via cellular filaments. The lens fiber cells exhibit a unique filamentous polymer, the so-called beaded filaments, when analyzed by electron microscopy. It is formed by a lens-specific cytoskeletal protein, referred to as CP49, and α-crystallins (Carter *et al.*, 1995). αA-crystallin also interacts with tubulin (Kato *et al.*, 1996) and actin (Wang and Spector, 1996). αA- and αB-crystallin block the depolymerization of the intracellular matrix component actin by cytochalasin D. However, phosphorylation of α-crystallin markedly decreases its protective effect. Moreover, there are several lines of biochemical evidence that α-crystallin might become associated with the plasma membrane. Obviously, actin would interact with lipid in this case. It is speculated that this interaction might protect and stabilize the lipid bilayer and decrease membrane permeability (Borchman and Tang, 1996).

The participation of α-crystallin in the stabilization and regulation of the cytoskeleton is additionally supported by recent findings in human trabecular meshwork. This tissue is distended and stretched with increasing intraocular pressure. Mitton *et al.* (1997) described that after a single 10% linear stretch of cells from a primary culture derived from human trabecular meshwork, αB-crystallin is degraded within the first 2 minutes by more than 1/4. Two hours after stretching, the *Cryab* mRNA concentration increases, indicating the recovery of αB-crystallin during reorganization of the cytoskeleton.

Diseases

In human and a variety of animal models at least three mechanisms are known to induce cataract formation: UV radiation, oxidative and osmotic stress. All these mechanisms lead to structural changes for the α-crystallins. Cys^{131} and Cys^{142} from αA-crystallin are present in transparent human lenses as a mixture of cysteine sulfhydryl and half-cysteine disulfide groups, while identical analysis from cataractous lenses demonstrated undetectable levels of the cysteine sulfhydryl group (Takemoto, 1996b). In human lenses an age-dependent increase of the α_{HM}-crystallin fraction can be observed as well as an increase of water-insoluble α-crystallin complexes. These decreases in soluble α-crystallin may indicate a decrease in protective capacity of the α-crystallins.

Because of the expression pattern of the α-crystallins, it is expected that several diseases should be caused or accompanied by alterations in α-crystallin. In the mouse, Brady *et al.* (1997) reported the consequence of the loss of the αA-crystallin gene in a knock-out mutation. The only pathological consequence found is an opacification of the lens. The targeted disruption of the αA-crystallin gene produced opacities resulting from inclusion bodies containing αB-crystallin. This finding is supported by the recent chromosomal localization of a new mouse lens opacity gene (*lop18*). This mutation was mapped on mouse chromosome 17, about 16 cM from the centromere, which makes it a likely candidate mutation for *Cryaa*. The homozygous mutants develop tiny vacuoles in the lens cortex at E14. These vacuoles are more prominent at E16, and the cataract continues to develop after birth. At the final stage 4 months after birth, there is prominent degeneration of the cortex, posterior migration of lens epithelial nuclei, and formation of abnormal lens fibers at the posterior pole of the lens (Chang *et al.*, 1996). Further molecular analysis of the *lop18* mutants should clarify whether the *lop18* is a mutant allele of *Cryaa* as well as explore the differences and similarities to the *Cryaa* knock-out mice.

Brady and Wawrousek also reported recently on the knock-out of the αB-crystallin encoding gene (1997). Interestingly, even the homozygotes displayed no overt mutant phenotype. Histological analysis of eyes, cardiac muscle and skeletal muscles from *Cryab*$^{-/-}$ revealed no apparent structural abnormalities detectable by light microscopy. Immunohistochemistry showed that αA-, β-, and γ-crystallins appear to be distributed normally. Even the double knock-out of both *Crya* genes led to viable and fertile mice with fully formed lenses. Epithelial cells appear normal in *Cryaa*$^{-/-}$/*Cryab*$^{-/-}$ lenses, but fiber cell organization seems to be altered by the absence of any α-crystallin.

αB-Crystallin, which is also expressed outside the lens, was shown to be associated with a broad variety of degenerative neurological diseases. First, αB-crystallin was found to be accumulated in scrapie-infected hamster brain cells (Duguid, 1988) and later in brains from humans having Creutzfeld-Jacob disease (CJD). In particular, the most intensive localization was observed in the spongiotic tissue representing progressively altered astrocytes in CJD. Under non-pathological conditions, αB-crystallin appears in the brain mostly in oligodendroglia cells (Renkawek *et al.*, 1992).

Furthermore, αB-crystallin accumulates in the brain of patients with Alexander's disease (Iwaki *et al.*, 1989). Alexander's disease is a rare sporadic encephalopathy characterized by macrencephaly, by extensive proliferation of abnormal astrocytes, and by formation of inclusions in astrocytes and their processes, so-called Rosenthal fibers. Since the findings on Alexander's disease in humans have been published, Weissenböck *et al.* (1996) reported a similar case in Bernese mountain dogs. Human and canine brains exhibited Rosenthal fibers, which were immunohistologically positive for αB-crystallin. It is currently assumed that the formation of Rosenthal fibers is part of a chronic stress response to an as yet unknown stimulus.

Additionally, a large number of αB-crystallin positive neurons was found in the cerebral cortices of patients with progressive supranuclear palsy. In particular, αB-crystallin positive ballooned neurons were frequently observed in deep cortical pyramidal cell layers of the limbic and paralimbic system in these diseases. The involvement of the limbic and paralimbic system may thus contribute to personality changes as well as to memory and cognitive impairment in these patients (Higuchi *et al.*, 1995). A dramatic increase of αB-crystallin expression has been shown in some different categories of human brain tumors: schwannomas, astrocytic tumors, meningoblastomas and chordomas. There is some experimental evidence that αB-crystallin overexpression in tumors coincides not only with the overexpression of hsp27, but also with the presence of estrogen receptor (Hitotsumatsu *et al.*, 1996). αB-Crystallin was demonstrated in about 10% of cases of diffuse Lewy body disease, a major cause of dementing illness. This may indicate a transient role for the αB-crystallin in the biogenesis of cortical Lewy bodies (Lowe *et al.*, 1990).

In the heart, αB-crystallin can be recovered under non-pathological conditions from the insoluble cell components. However, during an ischemic period, the cytosol of cardiomyocytes acidifies, thus causing the aggregation of the protein with cytoskeletal elements. The proportion of aggregated αB-crystallin increased in hearts reperfused after total normothermic ischemia of increasing severity. The αB-crystallin aggregation was found to be inversely correlated to the ability of the hearts to recover contractile activity after the ischemic episode, and can be used, therefore, as a new marker for the early onset of ischemic damage of the heart using biopsies of only a few milligrams (Chiesi and Bennardini, 1992; Barbato *et al.*, 1996).

β/γ-Crystallins

The β- and γ-crystallin polypeptides are recognized as members of a related β/γ-crystallin superfamily. According to the original finding of three main protein fractions in the eye lens (Mörner, 1893), the β-crystallins were characterized as oligomers (the molecular mass of the monomers is between 22 and 28 kDa) with native molecular masses ranging up to 200 kDa for octameric forms. Biochemically, the β-crystallins are also characterized by blocked N-termini. In contrast, the native γ-crystallin proteins are characterized as monomers with molecular weights of 20 kDa and a free N-terminus. β-Crystallin sequences differ from γ-crystallins mainly in having extensions at the N- and C-termini and in the conformation of the connecting peptide of the two domains. In β-crystallins, this connecting peptide is extended, whereas in γ-crystallin it takes a sharp turn. As a result, pairs of domains associate intramolecularly to form monomeric proteins in γ-crystallins, whereas in the β-crystallins the interaction is intermolecular, leading to oligomeric association (Zarina *et al.*, 1994). There is one protein that was earlier designated as βs-crystallin because of its slow chromatographic migration among the β-crystallin fraction and its blocked N-terminus. However, it can be found only as a monomer, and it is referred to today as γs-crystallin (van Rens *et al.*, 1989).

The common characteristic of all β- and γ-crystallins is the so-called Greek key motif (Figure 2). Crystallography has shown that each of the β- and γ-crystallins is composed of two domains built up by two Greek key motifs and it is widely accepted that β/γ-crystallins evolved in two duplication steps from an ancestral protein folded like a Greek key (for review see Lubsen *et al.*, 1988). In the β-crystallins, individual Greek key motifs are encoded by separate exons, whereas in the γ-crystallin genes two motifs are encoded by one exon reflecting the modular nature of the proteins during evolution. Indeed, a primary clue to the evolutionary origin of the β/γ-crystallin came from the NMR structural analysis of a yeast killer toxin from *Williopsis mrakii* (WmKT). This protein is considered an excellent candidate for a structural precursor for the entire β/γ-crystallin superfamily. The structural similarity that the one-domain WmKT bears to the γB-crystallin domain is

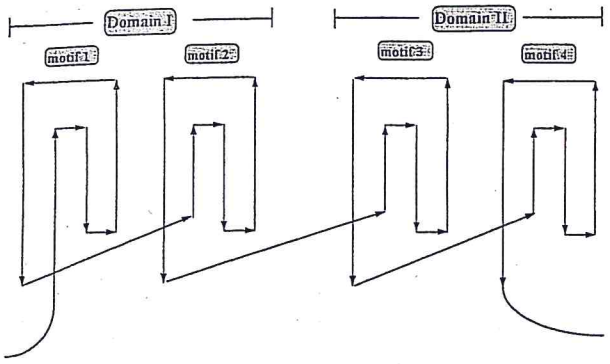

Fig. 2 Greek Key Motifs of β/γ-Crystallins.
The general structure of the 4 Greek key motifs in the β/γ-crystallin superfamily is schematically indicated. The motifs are built up by symmetrical, twisted antiparallel β-sheets. The N- and C-terminal extension varies among the subfamilies. In the β-crystallins, each motif is encoded by one exon, whereas in the γ-crystallin each exon encodes one domain consisting of two motifs.

not accompanied by any appreciable sequence homology. The 3D structural resemblance to the single β/γ-crystallin domain argues that it represents the stable fold that gave rise to the members of the superfamily, and diverged from some common one-domain ancestor. The WmKT protein is a secreted toxin of about 10 kDa and inhibits β-(1,3)-glucan synthetase. This toxin interfers with the formation of the cell wall in yeast (Antuch *et al.*, 1996). In this regard, it might be worth noting that some other relatives of β- and γ-crystallins, Protein S from *Myxococcus xanthus*, and spherulin 3a from *Physarum polycephalum* are involved in the formation of dehydrated spores. Another relative, the epidermis differentiation-specific protein (EDSP or EP37) of the amphibian *Cynops pyrrhogaster*, is expressed specifically in differentiating ectoderm (Wistow *et al.*, 1995). Three additional cDNAs were isolated and characterized recently as novel EP37 homologues. Two of them are exclusively expressed in the epidermis. During and after metamorphosis, the expression of EP37 proteins could be observed in cutaneous glands, and a transition to adult types of EP37 occurred (Ogawa *et al.*, 1997). Another non-lens member of the β/γ-crystallin superfamily was detected recently. Expression of this protein is altered in a model of tumor suppression of human melanoma. AIM1 (absent in melanoma) contains 12 Greek key motifs, suggesting a 6-domain structure (Ray *et al.*, 1997).

The function of this Greek key motif is actually unknown, however, recent computer-based analysis suggests that it might be responsible for particular protein-protein interactions similar to those seen with immunoglobulins (Crabbe and Goode, 1995). Like the immunoglobulins, the β/γ-crystallins are folded in an all β-structure. Undoubtedly, the accumulation of hydrogen bonds in the symmetrical twisted antiparallel β-sheet structure of each domain and the hydrophobic interactions between them contribute significantly to their stability. This overall stability can be understood as the sum of the contributions of independent folding units. The spatial correlation of the molecules at the given high concentration in the lens fiber cells could give rise to 'short-range order', which minimizes light scattering, thus providing optimum transparency of the eye lens (Jaenicke, 1994). The two domains of the γB-crystallin, indeed, are in agreement with this prediction (Mayr *et al.*, 1997).

β-Crystallins

Genes and Gene Expression

The family of β-crystallins can be divided into more acidic (βA-) and more basic (βB-)crystallins. Each subgroup is encoded by three genes (*Cryba1*, *-2*, *-4*; *Crybb1*, *-2*, *-3*), however, *Cryba1* encodes two proteins (βA1- and βA3-crystallin). This feature is conserved among all mammals, birds and frogs. In man, a second *Crybb2* gene was found; however, since no transcripts of this particular gene could be detected, it is referred to as a pseudogene (Brakenhoff *et al.*, 1992).

In mouse and man, the *Cryb*-genes are distributed among three chromosomes (Hulsebos *et al.*, 1995a, b); the *Cryba4* and all *Crybb* genes are located in one cluster (Table 2). The *Crybb2* gene, which might be considered as a typical β-crystallin encoding gene, consists, of 6 exons: the first exon is not translated, the second one encodes the N-terminal extension (Chambers *et al.*, 1995), whereas

Table 2 The β/γ-Crystallins

Gene	Chromosome		Protein
	Mouse*	Human	
Cryba1	11 (46)	17	βA1/A3-crystallin; 23/25 kDa
Cryba2	1 (41)	2	βA2-crystallin; 22 kDa
Cryba4	5 (59)	22q11.2–13.1	βA4-crystallin; 22 kDa
Crybb1	5 (59)	22q11.2–12.1	βB1-crystallin; 28 kDa
Crybb2	5 (60)	22q11.2–12	βB2-crystallin; 23 kDa
Crybb3	5 (60)		βB3-crystallin; 24 kDa
Crybψb2		22	Pseudogene
Crygs	?	3	γs-crystallin; 20 kDa
Cryga → Crygf	1 (32)	2q33–36	γA → γF-crystallin; 20 kDa

* cM position according to the Mouse Genome Database (1997) in brackets.

the subsequent four are responsible for one Greek key motif each. From an evolutionary point of view, the genes encoding the acidic β-crystallins can be grouped together, and are distinct from *Crybb* genes (Lu *et al.*, 1996). Based upon a complete set of sequences from chicken and bovine *Cryb* genes, Duncan *et al.* (1996a) pointed out that the duplication giving rise to the known vertebrate *Cryb* occurred over 300 million years ago. *Crybb2* and *Cryba1* are the most highly conserved *Cryb* genes. Interestingly, a head-to-head linkage of the *Cryba4* and *Crybb1* genes with a spacer of 2147 bp was found in chicken. The chicken *Cryba4* gene is the smallest *Cryb* gene ever described due to its small introns, which range in length from 68 to 96 bp (Duncan *et al.*, 1995a).

Although β-crystallins are expressed from early developmental stages in the eye lens, their expression continues and rises after birth so that the highest concentrations are usually found in the lens cortex. However, the expression pattern varies among the individual β-crystallins.

In a quantitative Northern blot study, Aarts *et al.* (1989) analyzed in detail the expression schedule of *Cryba1*, *Crybb2*, *Crybb3* genes in the rat. All *Cryb* genes investigated were expressed from E13 onward; the *Crybb3* reaches its maximal expression around birth and drops down to background level after 6 months; *Cryba1* transcripts are maximally present 2 months after birth and reach background level at the age of 8 months. The expression pattern for the other members of the acidic *Cryb* genes (*Cryba2* and *Cryba4*) investigated in calf lenses suggests that they have a similar transcription pattern as the *Cryba1* gene (van Leen *et al.*, 1987). In contrast, *Crybb2* expression increases until six months after birth, and the corresponding transcripts are present in remarkable amounts even after one year. The *Crybb1* is present only in the terminally differentiated fiber cells (van Leen *et al.*, 1987).

The translation efficiency of the various β-crystallin transcripts varies tremendously. For example, *Crybb1* and *Crybb2* transcripts are present at nearly the same amount in rat neonatal lenses, however, βB2-crystallin protein is not detectable, whereas βB1-crystallin is abundantly present (Aarts *et al.*, 1989). Recently βB2-crystallin was also found outside the lens in mouse and cat neural and pigmented retinas as well as in cat iris (Head *et al.*, 1995).

The molecular basis for expression of the *Cryb* genes has not yet been fully established; however, the current knowledge will be summarized briefly to demonstrate some common findings (Figure 3). For the chicken *Crybb1*, fragment −126/+30 is sufficient for expression in transfected chicken lens cells (Roth *et al.*, 1991), and a larger fragment −434/+30 is sufficient for lens-specific expression in transgenic mice (Duncan *et al.*, 1996b). Further footprint analysis identified two regions of this promoter (PL1 and PL2) capable of binding lens nuclear factors, and the mutagenesis of these elements reduced promoter activity in patched lens epithelial cells as well as in transgenic mice (Roth *et al.*, 1991; Duncan *et al.*, 1995b, 1996b). Recent data have indicated that Pax6 unexpectedly represses the activity of the chicken *Crybb1* and *Cryba1* promoters in cotransfection tests, leading to the suggestion of Pax6 as a negative regulator of the *Cryb* genes (Cvekl and Piatigorsky, 1996).

Because of its high level of expression among the β-crystallin encoding genes, *Crybb2* was investigated in more detail. In the mouse, promoter fragments of −275/+30 or −110/+30 are able to activate a reporter gene in a lens-derived cell line, but not in cells from non-lens origin. The TATA box is at the correct place, approximately 25 bp upstream from the transcriptional start point (Chambers *et al.*, 1995). The regulatory elements, which determine the activity of this gene in the rat, are located in a large distal region (−750/−123) and in the first intron. These regions together constitute an enhancer only during specific stages of differentiation. Otherwise, they act individually as silencers; another silencer element was located between −123/−77. A Pax6 binding site, which is found *in vitro*, is obviously not used *in vivo* (Dirks *et al.*, 1996b).

Among the genes encoding acidic β-crystallins, the *Cryba1* was investigated to some extent. The chicken *Cryba1* promoter fragment from −382/+22 is sufficient to drive a reporter gene in transfected primary lens epithelial cells of embryonic chicks, but further shortening to position −143 leads to a loss of this activity. However, both fragments (−382/+22 and −143/+22) direct reporter gene activity to the lens of transgenic mice. DNaseI footprints reveal binding of chicken lens nuclear proteins to two sites within the short −143/+22 fragment. The protected sequences exhibit similarities to some elements observed in the γ-crystallin promoters (McDermott *et al.*, 1996). In the region between −382 and −143 a complex enhancer element was recognized recently between −270 and −254 bp relative to the transcriptional start site. The length of a T-rich sequence downstream of the enhancer influences its activity. The enhancer region includes a consensus sequence for the basic region-leucine zipper proteins of the Ap-1 and CREB superfamily. Lens nuclear proteins

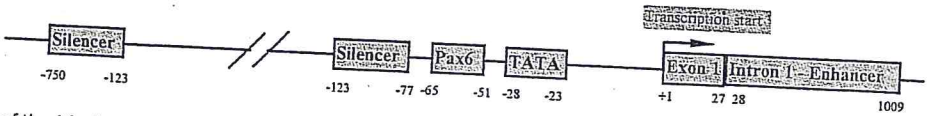

Fig. 3 Promoter of the Murine *Crybb2* Gene.
The elements of the *Crybb2* promoters depicted are those demonstrated to interact with proteins to regulate the expression of the corresponding gene. The localization of the various elements is given for the mouse promoter, however, in general, they are at similar positions at least in other mammals. The observed Pax6 binding site is not used *in vivo* to activate the *Crybb2* gene. For references refer to the main text (not drawn to scale).

bind the enhancer sequences to form several specific complexes, some of which are related antigenically to members of the AP-1 and CREB families of proteins (McDermott *et al.*, 1997).

Proteins and Functions

The native β-crystallin proteins can be found as multiple forms of oligomers with a general isoelectric point ranging between 5.7 and 7.0. The dimers, trimers and perhaps tetramers of β-crystallin subunits form a β-crystallin subfraction in gel chromatography called βL-crystallin (for low molecular mass; 40–100 kDa); larger aggregates (βH-crystallins; up to 200 kDa) are formed mainly by octamers. β-Crystallins are acetylated at their N-termini; further post-translational modifications are glycosylation and phosphorylation. By comparison of all available vertebrate *Crybb* sequences a putative protein-kinase C phosphorylation site immediately in front of the fourth Greek key motif was deduced for all basic β-crystallins, but not for the acidic ones (Zarbalis *et al.*, 1996). However, since *in vivo* phosphorylation was reported solely for the Ser^{230} in the βB2-crystallin (Kleinman *et al.*, 1988), this putative PKC-dependent phosphorylation and its possible function remains to be elaborated.

One interesting feature of β-crystallins is the association of βB2-crystallin as homodimers. This characteristic could be attributed to the connecting peptide between the two domains, each formed by two Greek key motifs. Additionally, removal of the flexible arms at the N or C terminus of βB2-crystallin results in the formation of dimers and tetramers, which could not be interconverted without denaturation (Trinkl *et al.*, 1994). From these studies it might be concluded that the linker is necessary for dimerization, whereas the N- and C-terminal arms appear to be involved in preventing the formation of higher homo-oligomers.

The N-terminal extensions, ranging from 10–58 amino acids, are specific for the β-crystallins and missing in the γ-crystallins. The largest extension is present in the βB1-crystallin; in the rat, it can be cleaved specifically by the protease calpain II leading to insolubility of the protein. In human lenses, a variety of additional N-terminal cleavage products could be observed mainly early in life (David *et al.*, 1996). In particular, a partially degraded form of the βA3/A1-crystallin missing either 22 or 4 amino acids from the respective N-terminal extension was detected (Lampi *et al.*, 1997). It leads to the hypothesis that such cleavages play important roles not only in the maturation process of the juvenile human lens but also in aging and in cataract formation.

Diseases

One of the inherited cataract mutants in mice, which has been well studied, is the Philly mouse. The mutation is inherited as an autosomal dominant trait (Kador *et al.*, 1980). The Philly lens lacks a functional mRNA responsible for the *Crybb2*. The corresponding cDNA from Philly lenses contains an in-frame deletion of 12 bp resulting in a loss of 4 amino acids. The region in which the deletion of the 4 amino acids occurs is close to the carboxy-terminus and essential for the formation of the tertiary structure of the βB2-crystallin (Chambers and Russell, 1991). Therefore, the morphological alterations have to be understood as consequences of the mutation in the *Crybb2* gene. The lenses develop normally until the first postnatal week. At that time, particles appear in the anterior cortex that extend by the 10th day to the anterior subcapsular area. A loss of the normal lens denucleation process and swelling of the lens fibers follows. The characteristic bow configuration of the nuclei is replaced by a fan-shaped configuration (Uga *et al.*, 1980). Faint anterior opacities seen at postnatal day 15 are followed by sutural cataracts at day 25, nuclear cataract at 30 days, lamellar perinuclear opacities at 35 days, and total nuclear cataracts with anterior and posterior polar involvement at 45 days. Cataractogenesis parallels in intralenticular increase in water, sodium, and calcium, and a decrease in potassium, reduced glutathione, and ATP. An altered membrane permeability is the cause of the increased outward leak of potassium (Kador *et al.*, 1980). The increasing severity of the phenotype is temporally correlated to the expression of the *Crybb2* gene.

Two human cataract mutations are currently thought to be associated to the β-crystallin encoding genes. Padma *et al.* (1995) reported a linkage of a gene causing a unique form of autosomal dominant zonular cataracts with Y sutural opacities to human chromosome 17q11–12 in a three-generation family. Since the *CRYBA1* gene is localized in this region, it is a good candidate for this particular disease. Furthermore, a cerulean blue cataract is described in a large family as a dominantly inherited disorder. This disease was closely linked to the region of human chromosome 22 that includes two β-crystallin encoding genes (*CRYBB2* and *3*) and a pseudogene (*CRYBB2ψ1*; Kramer *et al.*, 1996). Recently, a G→A transition was reported at the position of the first base of the codon normally coding for Glu residue 155 in *Crybb2*. This mutation creates a stop codon that truncates the βB2-crystallin by 51 amino acids (Litt *et al.*, 1997).

In addition, βB2-crystallin is also thought to be involved in age-dependent cataract formation. Takemoto (1997) could demonstrate that the formation of disulfide bonds between Cys^{37} and Cys^{66} can be observed only in human senile nuclear cataract (grade IV), but not in normal lenses. It remains to be established whether the disulfide bond formation of the two cysteine residues is the initial event that triggers denaturation of other parts of the protein, or if the cleavage from the N-terminal side could contribute to the loss of the native structure.

γs-Crystallin

The γs-crystallin might be understood as an ancestral intermediate between the β- and γ-crystallin family. The γs-crystallin gene (*Crygs*) is composed of three exons and

two introns. The first exon consists of 9 bp (carp) or 21 bp (bovine) and is followed by an intron of 4.25 kb. The exon 2 contains the information for 81 amino acids and exon 3 for 90 aa; the intermediate intron has a length of only 400 bp (van Rens *et al.*, 1989). The human *Crygs* gene was mapped using hamster-human hybrid cells to chromosome 3, it is obviously independent from other *Cryb* or *Cryg* genes (Table 2).

Only 75 bp of the *Crygs* promoter sequence immediately upstream of the transcription initiation site are known. No plausible CAAT box was found, whereas the TATA box is present and follows the common rules. The phylogenetic analysis revealed that the *Crygs* gene is more closely related to the other *Cryg* genes (see below) than to the *Cryb* gene family (van Rens *et al.*, 1989), even if the γs-crystallin has an acetylated N terminus and an isoelectric point at 7.0 (Rink *et al.*, 1982).

The bovine γs-crystallin is monomeric without any tendency to form assemblies up to concentrations in the millimolar range. The connecting peptide between the two domains has one extra amino acid residue compared with the βB2- and γB-crystallin; however, computer-based analysis demonstrated that in the γs-crystallin the charged residues are generally paired explaining the monomeric behavior of γs-crystallin by intramolecular associations (Zarina *et al.*, 1994).

The expression pattern of *Crygs* is unique among the *Cry* gene families because its transcripts cannot be detected during embryogenesis. During the first month after birth, *Crygs* transcripts accumulate in the lens and reach their maximal concentration after 2–3 months. It is expressed at a low level (20% of the maximum at three months) eight months after birth and seems to remain stable at this level (Aarts *et al.*, 1989).

One important function of the γs-crystallin in the eye lens might be in preventing phase separation of the cytoplasma of lens fiber cells. Such phase separation has been implicated in the formation of cataracts, where opacification of the eye lens results from the disturbances in the uniform spatial distribution of the lens proteins. Liu *et al.* (1996) demonstrated recently that γs-crystallin plays an important role in preventing this pathogenic mechanism. The presence of γs-crystallin in concentrated solutions lowers the phase-separation temperatures significantly. The γs-crystallin also suppresses the aggregation of γ-crystallin solutions, and these findings are consistent with the view that the presence of γs-crystallin in the lens helps to maintain its transparency.

γ-Crystallins

Genes and Gene Expression

The γ-crystallin encoding genes (*Cryg*) are organized as a cluster of six genes (γA → γF-crystallin: *Cryga* → *Crygf*) within approximately 50 kb in the rat (den Dunnen *et al.*, 1989). This gene cluster is found on mouse chromosome 1 (Skow *et al.*, 1988) and on human chromosome 2 (den Dunnen *et al.*, 1985; Table 2). The six genes are very similar and the protein sequences, which are deduced from the mouse *Cryge* and *Crygf* genes, are even identical (Graw *et al.*, 1991, 1993).

A typical *Cryg* gene is composed of three exons; the first exon contains only 9 bp and is followed by a short intron of about 100 bp. The second exon (243 bp) and the third exon (273–276 bp) are separated by a large intron (1–2 kb). The non-translated 3′-end is short (about 40 bp). The *Cryge* and *Crygf* genes, which are very strongly expressed in rodents during the late phase of gestation and in the juvenile phase, are not expressed in humans and, therefore, referred to as pseudogenes (Brakenhoff *et al.*, 1990).

The *Cryg* genes are expressed in mouse lenses from E13.5 onwards in the primary fibers and later on in the secondary fiber cells, but not in the epithelial cells (van Leen *et al.*, 1987, Santhiya *et al.*, 1995). The expression of *Cryg* genes reaches the maximum in mice at the first weeks after birth (Goring *et al.*, 1992). The decrease of *Cryg* expression could be correlated with an increase in methylation of the 5′-regions of various rat *Cryg* genes (Peek *et al.*, 1991). In human, the *Cryg* expression is restricted to the prenatal development, because of the different time scale of mouse and human intra-uterine life.

In contrast to mammals, γ-crystallins appear in amphibians first, even before the α-crystallins. Immunofluorescence studies and Western blot analysis have shown that in *Xenopus Cryg* genes are expressed initially in the lens placode stage (McDevitt and Brahma, 1973; Shastry, 1989). More recently, Smolich *et al.* (1994) reported that in *Xenopus* some *Cryg* genes are expressed at low levels outside the lens during gastrula and neurula stages indicating that this early expression is not a response to initial determinative events in lens induction and is not restricted to presumptive head ectoderm. Also, non-lens expression of *Cryg* mRNA is not restricted to early embryogenesis but continues in other differentiated tissues present by tadpole stages in middle and posterior regions. Therefore, it might be suggested that the γ-crystallins play a role in general cellular processes in *Xenopus*.

The promoters for the very similar *Crygd*, *Cryge* and *Crygf* genes have been intensively studied in mouse and rat (Figure 4). In the mouse, previous studies have established that the 5′-flanking region −226/+45 of the *Crygf* crystallin gene is sufficient for optimal promoter activity in the lens and that the proximal element (−67/−25) is sufficient to elicit lens-specific gene expression (Lok *et al.*, 1989; Liu *et al.*, 1991; Goring *et al.*, 1993). Elimination of the retinoic acid response element at −210/−185 (RARE; Tini *et al.*, 1993), also designated as hormone response element HRE (Tini *et al.*, 1995), results in a dramatic decrease in activity approaching background level. This is due to a response to the ligands of the $RAR\alpha_1$ and/or $RAR\beta_2$ receptors. Cotransfection with recombinant $RAR\alpha_1$ and $RAR\beta_2$ receptors enhanced the activity of the γF-

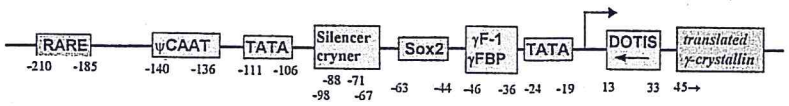

Fig. 4 The Promoter of Rodent *Crygdef* Genes.
The promoters of the mouse and rat *Crygdef* genes are very similar, and most of the sequence elements can be identified in all of them. The region −226/+45 is sufficient to elicit lens specific gene expression; identified elements within this region are depicted schematically. For details and references refer to the main text (not drawn to scale).

crystallin promoter 25-fold and this was mediated by the RARE element.

The fragments −171/+45 and −67/+45 of the murine γF-crystallin promoter exhibited no detectable reporter gene activity in N/N1003A lens cells. However, they lead to a lens-specific expression of reporter genes in transgenic mice (Yu *et al.*, 1990; Goring *et al.*, 1993). This might be explained by the activation by Sox2 (−63/−44; Kamachi *et al.*, 1995).

Sequence comparison of all rat, mouse and *Cryg* promoter sequences revealed a common inverted repeat structure at −98/−67. This particular structure was referred to as *Cryner* (**Cryg ne**sted **r**epeats) and suggested to be a strong candidate for DNA-protein interaction (Graw *et al.*, 1993). Indeed, recent studies from our laboratory demonstrated that lenticular proteins interact with these structures, however, only with the *template* strand; the responsible protein has not yet been identified (Stöger *et al.*, 1997).

For the rat γD-crystallin promoter a silencer element spanning −85 to −67 is active in non-lens cells (Peek *et al.*, 1992). Recently, its function was extended to lens cells during βFGF-mediated lens fiber differentiation *in vitro*. In late differentiated fiber cells endogenous and exogenous γD-crystallin expression was down-regulated, and a repressor protein protected the silencer element (Dirks *et al.*, 1996a). Furthermore, DNA-protein interaction studies revealed a novel zinc-finger protein binding to the γF-1 element (−46/−36). However, functional studies showed that the corresponding binding protein γFBP inhibits the activation of the γF-crystallin promoter in chicken lens cells (Liu *et al.*, 1994).

Interestingly, *in vitro* experiments revealed that α-crystallins interact with an element positioned immediately downstream of the transcriptional initiation site of the γE-crystallin gene (+33 → +13), referred to as DOTIS (Pietrowski *et al.*, 1994). Previous reports dealing with γ-crystallin promoter activity with deletions at equivalent positions of the rat γD- and murine γF-crystallin promoters revealed reduction of the promoter activity (Peek, 1990; Lok, 1989). These deletions implied that the 3′-nucleotides from +18 to +12 (*template* strand) are necessary for full transcriptional activity. Recently, it could be demonstrated that autophosphorylation of α-crystallin leads to a loss of its ability to interact with the DOTIS element; however, this effect was not observed if the α-crystallins were phosphorylated by PKA (Pietrowski and Graw, 1997). Unpublished observations from our laboratory pointed out that recombinant αA-crystallin enhances the *Cryge* promoter activity in a transient reporter gene assay only in the presence of the DOTIS element.

Recent data suggest that Pax6 is an important factor in gene activation during eye and lens development. However, no Pax6 binding site could be detected in the rat *Crygd* promoter (Dirks, 1996a), or in the mouse *Cryge* promoter (Krausz and Graw, unpublished). Therefore, it might be concluded that Pax6 is responsible for early expression of *Cryaa* and *Cryab* genes, but not for the later expression of *Cryg* genes.

Proteins and Function

γ-Crystallins appear in the gel chromatography of lens proteins as the last peak because of their low molecular mass of 20 kDa and of their monomeric structure. They are the most basic crystallins in mammals, with isoelectric points ranging from 7.1 to 8.6 (Rink *et al.*, 1982). Furthermore, γ-crystallins are characterized by a high content of free Cys residues (4–7 per molecule). X-ray crystallography of the calf γB-crystallin has demonstrated that the spatial localization of Cys^{18} and Cys^{22} in the tertiary structure permits disulfide bond formation; however, in the other bovine γ-crystallins the Cys^{22} is replaced by His. Indeed, reduction of the total γ-crystallin fraction with DTT resulted in an increase of approximately 2.5 mol of free SH per mole γB-crystallin (McDermott *et al.*, 1988). The high number of free SH groups makes the γ-crystallins very sensitive to the formation of mixed disulfides during oxidative stress. It is widely accepted that the formation of mixed disulfides with reduced glutathione (GSH) should prevent aggregation of γ-crystallin with itself or with other proteins and keep the γ-crystallins in solution. The lens has a relatively high GSH content, and more than 80% of the thiol content of the tissue is found as protein thiol located predominantly in the β- and γ-crystallins. Under normal conditions, the ratio of GSH to GSSG in the lens is about 100:1, and about 10% of the GSH is found bound to proteins. Experiments in lens organ culture under increasing oxidative stress induced by *tert*-butylhydroperoxide resulted in increased protein-GSH mixed disulfide formation. Bovine γB-crystallin was demonstrated under such conditions to react with up to three GSH equivalents to form mixed disulfides associated with minor conformational changes (Willis and Schleich, 1996).

DNA and protein sequence studies indicated for mouse, rat and man that the 6 γ-crystallins can be clustered into two groups of γA/B/C-crystallin and γD/E/F-crystallin (Siezen *et al.*, 1987; 1988; Graw *et al.*, 1993). It might be

noteworthy that this clustering reflects differences in their behavior in phase separation. The critical temperature at which phase separation occurs is low for the γA/B/C-crystallins and high for the γD/E/F-crystallins. This finding might be explained by distinct states of hydration observed between members of the two groups. The difference in hydration state could be attributed to three amino acid residues in the γD-crystallin (Leu^{51}, Ile^{103} and His^{115}), where there was a change of hydrophobicity/hydrophilicity compared with γB-crystallin (Slingsby *et al.*, 1997).

Diseases

Lens genetics research identified various mutations in the *Cryg* genes. Currently, in mice eight independent mutations were determined to be associated with the *Cryg* gene cluster. The *Elo* mutant (*Eye lens obsolescence*) was characterized as a single nucleotide deletion in the *Crgye* gene. The mutation destroys the reading frame of the gene, and at the protein level, the fourth and last Greek key motif of the protein (Cartier *et al.*, 1992). The allelic series of the Neuherberg *Cat2* mutants was also demonstrated to be closely linked to the *Cryg* cluster (Löster *et al.*, 1994). At first, 5 independently observed and phenotypically distinct mutant lines were grouped to this allelic series (Kratochvilova and Favor, 1992), but just recently two further mutants were characterized as members of the *Cat2* group (Everett *et al.*, 1994). Recent results from our laboratory define $Cat2^{nop}$ as a combined deletion and insertion of 11 and 4 bp, resp., in the *Crygb* gene, and $Cat2^{ns}$ as a deletion of the entire 3′ end of *Cryge*.

Therefore, the various phenotypes in the *Elo* and *Cat2* mutants have to be reanalyzed in the view of consequences of mutations in the *Cryg* genes. The alterations are instructive about the functions of the γ-crystallins. These mutants have a block in the maturation of primary lens fibers and in the differentiation of secondary lens fibers. The elongation of the secondary fiber cells is not mature and they do not reach the poles of the lens. In the case of the $Cat2^{ns}$ mutants the phenotype is manifested as a suture cataract in the heterozygotes (Graw *et al.*, 1989) and in the case of the $Cat2^{nop}$ mutants as a nuclear cataract. The nuclei in the primary and secondary fiber cells are not completely degraded in either of these cataracts (Graw *et al.*, 1984; 1990).

In the *Elo* mouse, the first change was detected on E12.5. Elongation of the central fibers at the basal cytoplasm was impaired. Necrotic cells were found among the central lens fibers, which never reached full maturation length and progressively degenerated thereafter (Oda *et al.*, 1980). This phenotype seems to be more severe than the $Cat2^{nop}$ phenotype. In this particular mutant, the decreased expression of *Cryg* genes can be observed from E12, however, morphological alterations can be found by E15 (Santhiya *et al.*, 1995).

In humans, a mutation re-activating the human *Cryge* pseudogene was identified leading to the so-called 'Coppock-like' cataract. The Coppock-like cataract is virtually nonprogressive and affects only the embryonic nucleus of the lens leading to a very mild phenotype. A cluster of sequence changes was found within and around the TATA box leading to its reactivation (Brakenhoff *et al.*, 1994). The pseudogene has an in-frame stop codon and thus codes for a 6 kDa polypeptide truncated in the middle of the second motif (Meakin *et al.*, 1987).

In addition to genetically determined cataract formation, lens opacification is a common occurrence with aging. There is considerable epidemiological and experimental evidence to suggest that the maintenance of tissue optical clarity is in part dependent on thiol redox chemistry. In models of oxidative cataractogenesis and in most human senile cataracts, GSH and free protein thiol levels decline with commensurate increases in protein thiol oxidation. Since the γ-crystallins are highly susceptible to the formation of mixed disulfides because of their high number of SH groups, they may be predominantly involved in cataracts caused by oxidation possibly leading to cross-linking.

One cause for oxidative stress might be UV light, since the eye is exposed to this light in various intensities throughout life. UV light leads to photo-oxidative damage in the lens in a variety of experimental systems, and this photo-oxidative challenge would also affect the γ-crystallins. Of particular interest might be the combination of these two factors, increased environmental UV burden (Klein *et al.*, 1995) and a genetic predisposition to cataract. Each of these alone might have only a small effect on the lens; however, combining the effects, cumulative alterations might be expected. This can be observed experimentally using the mouse mutant $Cat2^{ns}$, which has only a faint suture cataract as heterozygotes (Graw *et al.*, 1989), caused by a deletion of the 3′ end of *Cryge*. After UV treatment, the severity of the cataract increases to be equivalent to that observed in the homozygotes (Forker *et al.*, 1997). This set of experiments demonstrated for the first time the possibility of differentiating between endogenous (genetic) and exogeneous (environmental) effects in cataractogenesis and allows a detailed investigation of the dual function of γ-crystallins in these processes.

Taxon-Specific Crystallins

Evolutionary analysis of the classical α-, β- and γ-crystallins indicated their relationship to other proteins with distinct functions outside the lens. However, additional proteins were found to be expressed at high levels in the lenses of some species; for example, the chicken δ-crystallin has close similarity to argininosuccinate lyase and the duck ε-crystallin to lactate dehydrogenase. Analyzing a broad variety of animals revealed a long list of species-specific crystallins, which were obviously recruited from enzymes and expressed in the lens up to 10–20% of the water-soluble protein. The concept of recruitment of lens crystallins by gene sharing from enzymes, which are active outside the lens, was described first be Wistow and

Piatigorsky (1987) and further elaborated by Wistow (1993b).

An interesting case in particular to human disease is the ξ-crystallin found at first in the guinea pig (Huang *et al.*, 1987), and later in camels (Garland *et al.*, 1991) and cattle (Rao *et al.*, 1997). This enzyme-crystallin is structurally related to alcohol dehydrogenase (Borras *et al.*, 1989) and has quinone oxidoreductase activity (Rao *et al.*, 1992). The recruitment of this enzyme as a crystallin can be explained by the use of a lens-specific alternative promoter, which does not require host-specific factors. The strong lens-preference of this promoter was demonstrated in both cultured cell transfections and in transgenic mice. The ZPE (ξ-crystallin protected element) at position −202/−152 in the *Cryz* promoter is important for its lens-specific expression (Lee *et al.*, 1994) and was further identified as a Pax6 binding site (Richardson *et al.*, 1995).

The ξ-crystallin belongs to a superfamily of medium-chain dehydrogenases/reductases (MDR) including the quinone reductase, glucose dehydrogenase and alcohol dehydrogenase. The ξ-crystallin itself has an oxidoreductase activity and requires NADPH as a cofactor, but it lacks a Zn-binding site. The amino acid sequence demonstrates that guinea-pig ξ-crystallin contains 5 thiol groups/subunit (like the γ-crystallins; Borras *et al.*, 1989; Rao *et al.*, 1992). The ξ-crystallin occurs as a tetrameric protein in the lens (Edwards *et al.*, 1996), and its oxidoreductase activity can be inhibited by the anti-coagulant dicoumarol (Duhaiman, 1996a) or by juglone in a competitive manner (Duhaiman, 1996b), as well as by reducing agents indicating the involvement of a disulfide bridge in its catalytic center (Duhaiman and Rabbani, 1996). The recently characterized bovine ξ-crystallin (Rao *et al.*, 1997), however, has distinct functional characteristics. Besides 83% sequence identity and similar physical and chemical properties, it shows minimal quinone oxidoreductase activity, but a strong binding affinity to single-stranded DNA. The authors attributed this difference to the exchange of Tyr^{59} (guinea pig) to a His in the corresponding bovine form.

It is noteworthy that an autosomal dominant cataract line in guinea pig (13/N) has been characterized by a dinucleotide deletion at the acceptor splice site of intron 6 of *Cryz*. The mutation results in elimination of exon 7 during mRNA processing, which in turn results in the altered ξ-crystallin protein missing 34 amino acids (Rodriguez *et al.*, 1992). This finding explains the altered structural and enzymatic properties of ξ-crystallin in guinea pig hereditary cataracts (Rao and Zigler, 1992). ξ-Crystallin is also expressed in the human lens (Gonzalez *et al.*, 1995) and the encoding gene *CRYZ* could be assigned recently to human chromosome 1p22–31. It has been suggested that this linkage permits the evaluation of the role of *CRYZ* in human cataractogenesis (Heinzmann *et al.*, 1994).

Acknowledgement

This work was supported at least in part by the European Union concerted action on the Role of Membranes in Lens Ageing and Cataract (contract No BMH1-CT93-1650) and by a grant from the Deutsche Forschungsgemeinschaft (DFG; Gr 1036/4-1). Stimulating discussions with Drs. Thomas Immervoll (GSF-Institute of Mammalian Genetics, Neuherberg, Germany) and Paul Russell (National Eye Institute/NIH, Bethesda, Maryland, USA) are gratefully acknowledged.

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