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Gene duplication and separation of functions in *α*B-crystallin from zebrafish (*Danio rerio*)

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Abstract

We previously reported that zebrafish αB-crystallin is not constitutively expressed in nervous or muscular tissue and has reduced chaperone-like activity compared with its human ortholog. Here we characterize the tissue expression pattern and chaperone-like activity of a second zebrafish αB-crystallin. Expressed sequence tag analysis of adult zebrafish lens revealed the presence of a novel α-crystallin transcript designated *cryab2* and the resulting protein αB2-crystallin. The deduced protein sequence was 58.2% and 50.3% identical with human αB-crystallin and zebrafish αB1-crystallin, respectively. RT-PCR showed that αB2-crystallin is expressed predominantly in lens but, reminiscent of mammalian αB-crystallin, also has lower constitutive expression in heart, brain, skeletal muscle and liver. The chaperone-like activity of purified recombinant αB2 protein was assayed by measuring its ability to prevent the chemically induced aggregation of α-lactalbumin and lysozyme. At 25 °C and 30 °C, zebrafish αB2 showed greater chaperone-like activity than human αB-crystallin, and at 35 °C and 40 °C, the human protein provided greater protection against aggregation. 2D gel electrophoresis indicated that αB2-crystallin makes up ≈0.16% of total zebrafish lens protein. Zebrafish is the first species known to express two different αB-crystallins. Differences in primary structure, expression and chaperone-like activity suggest that the two zebrafish αB-crystallins perform divergent physiological roles. After gene duplication, zebrafish αB2 maintained the widespread protective role also found in mammalian αB-crystallin, while zebrafish αB1 adopted a more restricted, nonchaperone role in the lens. Gene duplication may have allowed these functions to separate, providing a unique model for studying structure–function relationships and the regulation of tissue-specific expression patterns.

Keywords

crystallins; heat shock proteins; lens; molecular chaperones; zebrafish

The α-crystallins are evolutionarily related members of the small heat shock protein (sHSP) superfamily which are taxonomically ubiquitous components of the vertebrate eye lens [1].

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The αA-crystallin and αB-crystallin genes arose through a gene duplication event that occurred early in vertebrate history and are most closely related to sHsp20 [2]. Mammalian αA-crystallin is primarily lens specific and has lost the stress induction response that characterizes most sHsps, although some metals induce its expression [3]. In contrast, multiple cellular stresses induce mammalian αB-crystallin expression in a variety of tissues [4]. The mammalian α-crystallins act as chaperone-like molecules by binding to and preventing the aggregation of non-native proteins [5]. In addition, some studies suggest that they are true chaperones which can also aid in protein refolding [6].

The mechanism behind the chaperone-like activity of α-crystallin is of great interest because protein aggregation is believed to play a prominent role in the etiology of lens cataracts, a leading cause of human blindness. Temperature has a large influence on the ability of α-crystallin to inhibit protein aggregation. For example, raising incubation temperature increases the chaperone-like activity of mammalian α-crystallin hetero-aggregates [7] and both homoaggregates [8]. Temperature may influence chaperone-like activity by altering surface hydrophobicity [7,9–11], subunit exchange [12–14], or overall protein stability [15]. Increasing temperature also activates a higher-affinity binding mode in mammalian αB-crystallin [16]. As mammals maintain a relatively stable body temperature, they are not suitable for determining how α-crystallins are evolutionarily modified to function at different temperatures. Examining vertebrate species with different physiological temperatures can provide insights into the relationship between α-crystallin structure and function.

Studies suggest that α-crystallins adapt to diverse physiological temperatures. For example, the thermal stability of native α-crystallin correlates with the species' physiological temperature [17,18]. In addition, the thermal stabilities of recombinant zebrafish αA-crystallin and αB-crystallin are each lower than their respective human orthologs [19]. Chaperone-like activity also varies between zebrafish and human α-crystallins. Zebrafish αA-crystallin shows greater chaperone-like activity at lower temperatures than its human ortholog, suggesting that its protective function has been shifted to lower temperatures [19]. These data suggest that zebrafish α-crystallins have adapted to the lower body temperature of this species than mammals.

Zebrafish αB-crystallin has diverged far more in structure, expression and function from human αB-crystallin than have zebrafish and human αA-crystallin. For example, zebrafish αA-crystallin exhibits lens-specific expression that is similar to the mammalian expression pattern [20]. In contrast, zebrafish αB-crystallin expression is restricted to the lens, whereas its mammalian orthologs are also expressed in neural and muscle tissues [21]. Furthermore, the chaperone-like activity of zebrafish αB-crystallin is greatly reduced compared with the human protein [19]. Reduced expression and function in a zebrafish protein compared with its mammalian ortholog is not unusual. Ray-finned fishes experienced a genome-wide duplication event early in their evolution, and many single-copy mammalian genes are found as functional duplicates in extant fishes. Often the function and expression pattern of the original single-copy gene is divided between the duplicated copies [22–24]. The restricted expression and reduced chaperone-like activity in zebrafish αB-crystallin suggest the presence of a second ortholog in this species.

In this study we report the identification and characterization of a second αB-crystallin in zebrafish (αB2). The protein possesses only 50.3% amino-acid identity with the previously identified zebrafish αB-crystallin (αB1). Zebrafish αB2 is more widely expressed than αB1, being found in multiple tissues including lens, muscle and brain. Furthermore, recombinant αB2 exhibits strong chaperone-like activity, in contrast with the lower activity of αB1. Collectively, these data indicate that the two zebrafish αB-crystallins are under divergent

selection pressures and probably play different physiological roles. The presence of two zebrafish αB-crystallins differing in structure, chaperone-like activity and spatial expression provides a unique model for studying structure–function relationships and the regulation of tissue-specific expression patterns.

Results

Gene and protein sequence

As part of the NEIBank project for ocular genomics, cDNA libraries from zebrafish adult eye tissues were created and used for expressed sequence tag analysis. The unnormalized lens library was particularly rich in cDNA clones for several γ-crystallins [25], but among the most abundant clones sequenced were three clusters of cDNAs for members of the α-crystallin family. From a total of about 3700 sequences, 63 corresponded to αA-crystallin and 24 to αB-crystallin. However, a third group of 28 clones corresponded to a second αB-like gene. Single additional clones for this gene were also found in a whole eye library and in a library derived from posterior segment minus retina. Three different polyadenylation sites were identified within these transcript sequences, with the longest transcript of 2195 bp (GenBank accession No. DQ113417). The sequence matched a previously identified but unannotated zebrafish sequence (BC076518) and an unannotated genomic sequence from chromosome 21 (BX510931). The ORF encoded a protein sequence of 165 amino acids (Fig. 1; AAZ15808). Sequence comparisons showed that the predicted protein sequence was most closely related to αB-crystallins, and the novel protein and gene were named αB2-crystallin and *cryab2*, respectively. Interestingly, the zebrafish αB2 amino-acid sequence was more similar to human αB-crystallin (58.2%) than to zebrafish αB1-crystallin (50.3%).

Figure 1 shows the alignment of the two zebrafish αB-crystallin protein sequences with those for catfish and human αB-crystallin. Zebrafish αB2 contains two deletions and one insertion not found in the two other fish proteins but shares two of the three serine phosphorylation sites present in bovine αB-crystallin, while zebrafish αB1 contains only one. The arginine at position 120 in the human sequence, which is vital to chaperone-like activity, is present in all three fish proteins [26,27]. However, the three fish αB-crystallins (zebrafish αB1, αB2 and catfish αB) show variation in three of the eight amino-acid residues identified by Sharma *et al*. [28] as a chaperone-binding site in bovine αB-crystallin, and all three fish proteins show substantial variation in their C-terminal extensions. Pasta *et al*. [29] identified a nine-amino-acid sequence that, when deleted, reduces stability and increases chaperone-like activity of human α-crystallins. Zebrafish αB2 contains a four-amino acid deletion in this region. Phylogenetic analysis confirmed that, although zebrafish αB1 and αB2 both cluster with αB-crystallin sequences of mammal and bird species and are distinct from αA-crystallin and other sHSPs, they are strikingly divergent from each other (Fig. 2). Furthermore, the zebrafish αB-crystallins have diverged more from their orthologs in mammals and birds than zebrafish αA-crystallin has diverged from its orthologs.

Tissue-specific expression

Zebrafish αB1 is not constitutively expressed in neural or muscle tissue, but has so far only been identified in the lens [21]. We examined the tissue-specific expression of the novel αB-crystallin by semiquantitative RT-PCR and found that zebrafish αB2 is constitutively expressed in multiple tissues (Fig. 3). Expression was highest in the lens, moderate in brain, heart and skeletal muscle, and lowest in the liver, which is similar to mammalian orthologs. The slightly reduced expression levels of the tubulin control in the lens and skeletal muscle samples may be due to reduced amounts of total RNA in these samples. As these two tissues produced strong zebrafish αB2 products, the reduction in the tubulin control amplification products does not complicate the interpretation of these data. Of 35 estimated sequence tags

for zebrafish αB2 in GenBank (UniGene Dr32019), one is derived from pectoral fin, four from whole body, and all the others from lens or other eye libraries.

2D gel electrophoresis was performed to quantify the relative amounts of zebrafish α-crystallins in the lens. A single spot was identified as zebrafish αB2 by comparing its position with a sample of recombinant zebrafish αB2 run in parallel (Fig. 4; parallel recombinant protein not shown). Densitometry indicated that zebrafish αB2 comprised ≈0.16% of the total lens protein. A single spot containing both zebrafish αB1 and αA-crystallin was identified by comparing its position with a sample of recombinant proteins run in parallel, as well as probing with a polyclonal antibody to zebrafish αB1. The production of this antibody is described in Dahlman *et al*. [19] and was previously shown to react with both zebrafish αB1 and αA-crystallin. Densitometry indicated that this combined spot made up 2.18% of the total protein content of the lens. Because zebrafish αB1 and αA-crystallin have similar isoelectric points and molecular masses, it was not possible to distinguish them on the 2D gel. Zebrafish αB1 has the most acidic isoelectric point (5.7) of any known αB-crystallin. Two spots to the left of the combined zebrafish αA and αB1 spot are possible modifications of α-crystallins (Fig. 4). Modifications in mammalian α-crystallins such as phosphorylation make the proteins more acidic. In addition, these spots reacted with the polyclonal antibody described above (data not shown). A spot that is smaller in molecular mass than the three identified α-crystallins may be a truncation product.

Protein expression and chaperone-like activity

An expression construct containing the entire coding region for zebrafish αB2 was used to produce recombinant protein. The protein produced had a smaller molecular mass than the other two zebrafish α-crystallins, as predicted from its sequence (Fig. 5). Some minor bacterial protein content could not be removed during the purification procedure. MS confirmed that, like mammalian αB-crystallins, both zebrafish αB-crystallins contain an N-terminal methionine (data not shown).

Previous work demonstrated that zebrafish αB1 has reduced chaperone-like activity compared with its human ortholog [19]. In this study we examined the ability of zebrafish αB2 to suppress the chemically induced aggregation of α-lactalbumin and lysozyme at temperatures of 25–40 °C. At 27 °C, the physiological temperature for the zebrafish, αB2 showed greater chaperone-like activity than human αB-crystallin with either target protein (Fig. 6). However, at human physiological temperature (37 °C), the human ortholog provided greater protection against aggregation. Zebrafish αB2 also exhibited greater chaperone-like activity at 25 °C and 30 °C against the aggregation of α-lactalbumin, while human αB-crystallin displayed greater activity at 35 °C and 40 °C (Fig. 7). These differences in activity were significant at 25 °C ($P < 0.001$) and 40 °C ($P < 0.01$), but not at 30 °C or 35 °C. Differences between human αB and zebrafish αB1-crystallin were significant at all temperatures ($P < 0.05$). Differences between zebrafish αB1 and αB2 were significant at 25 °C ($P < 0.001$) and 30 °C ($P < 0.001$), but not at 35 °C or 40 °C.

Discussion

Zebrafish (*Danio rerio*) is the first species known to express two different αB-crystallins. We previously characterized a zebrafish αB-crystallin (αB1) that is lens specific and has lower chaperone-like activity than human αB-crystallin [19,21]. The novel protein described in this study (αB2), however, is expressed both within and outside the lens (Fig. 3) and exhibits higher chaperone-like activity than its human ortholog within the zebrafish physiological temperature range of 25–30 °C (Figs 6 and 7). Thus, zebrafish αB2 displays the more widespread tissue expression pattern that characterizes the mammalian αB-crystallins and possesses a more functionally appropriate level of chaperone-like activity

than zebrafish αB1. The clustering of zebrafish αB2 with tetrapod αB-crystallins in our phylogenetic analysis (Fig. 2) also shows that its structure has been more highly conserved than that of αB1. The lack of a second αB-crystallin in tetrapod taxa and the occurrence of a genome duplication event early in ray-finned fish evolution [24] suggest that the two zebrafish αB-crystallin genes arose within the ray-finned fish lineage. Therefore, the two αB-crystallins are paralogs of each other, and both are orthologs to the single gene found in mammals [30].

Multiple differences between the two zebrafish αB-crystallins suggest that they have evolved to play different physiological roles since their divergence possibly 200–450 million years ago. First, the two zebra fish αB-crystallins share lower amino-acid identity (50.3%) than either does with its human ortholog (60% and 58.2%). As the zebrafish proteins are more closely related to each other evolutionarily than either is to the human protein, this low identity is not reflective of genetic distance and suggests that selection pressures have caused the protein divergence. Second, the two zebrafish αB-crystallins exhibit different tissue expression patterns. Assuming that the ancestral gene was expressed throughout the body, like the single-copy mammalian version, zebrafish αB1 evolved a more restricted expression pattern. Third, the two zebra fish αB-crystallins exhibit different levels of chaperone-like activity, with αB2 possessing a greater ability to prevent protein aggregation than the αB1 paralog. Strong chaperone-like activity in both mammalian αB-crystallin and zebrafish αB2 suggests that a strong chaperone role was present in the ancestral zebrafish protein, and was lost during the evolution of zebrafish αB1. The evolutionary conservation of both gene copies, divergence in tissue expression pattern, and difference in chaperone-like activity all suggest that the functions typical of mammalian αB-crystallins are divided between the two zebrafish proteins. Similar sub-functionalization in zebrafish genes after duplication has been identified in cellular retinoic acid-binding proteins [23]. Separation of functions after gene duplication also occurred during evolution of δ-crystallin, a major component of the bird and reptile lens, from the enzyme argininosuccinate lyase (ASL). After duplication of the *ASL* gene, δ1-crystallin lost enzyme activity and became restricted to the lens, whereas δ2-crystallin retained its enzymatic activity and widespread expression pattern [31,32].

The zebrafish α-crystallins have adapted to function at zebrafish physiological temperature, which is lower than that of mammals. For example, zebrafish αB2 provides greater protection against aggregation at lower temperatures than human αB-crystallin, but less protection at higher temperatures (Fig. 7). This is similar to zebrafish αA-crystallin, which exhibits equivalent chaperone-like activity at its physiological temperature of 27 °C to the human ortholog at 37 °C [19]. This shift of chaperone-like activity to lower temperatures may provide suitable protection against protein aggregation at the zebrafish's body temperature. These thermal shifts in chaperone-like activity may reflect the need for enzymes to strike a balance between maintaining sufficient flexibility for molecular interactions, while maintaining enough structural stability to prevent denaturation [33]. Van Boekel *et al*. [15] have applied this concept to the chaperone-like function of mammalian α-crystallins, showing that bovine αA-crystallin is more thermally stable than αB-crystallin while exhibiting lower chaperone-like activity at equivalent temperatures. If α-crystallins balance the need for both flexibility and stability, one would expect this balance to shift in species with different physiological temperatures. In fact, zebrafish αA-crystallin exhibits both increased chaperone-like activity at lower temperatures and decreased thermal stability relative to mammalian αA-crystallin [19]. In addition, although thermal stability was not examined in the present study, the chaperone-like activity of zebrafish αB2 has shifted to lower temperatures. Interestingly, the chaperone-like activity of zebrafish αB2 fell as temperatures increased towards 35 °C (Fig. 7). In contrast, zebrafish αA-crystallin, zebrafish αB1-crystallin and both human α-crystallins generally interact with non-native protein more

effectively as temperature increases [19]. Multiple variations in primary structure may contribute to the observed differences in chaperone-like activity and thermal stability between α-crystallins (Fig. 1). Future studies can address the structure/function relationships and molecular mechanisms behind thermal shifts in chaperone-like activity.

Yu *et al*. [34] analyzed the chaperone-like activity and thermal stability of an αB-crystallin from the catfish *Clarius batrachus* (AAO24775). The catfish αB-crystallin exhibits strong chaperone-like activity similar to our findings for zebrafish αB2. In addition, the catfish protein shows greater amino-acid sequence identity with zebrafish αB2 than zebrafish αB1 (64.4% versus 57%), and a phylogenetic analysis grouped the catfish protein with zebrafish αB2 (Fig. 2). Thus, the aminoacid sequence analysis suggests that the catfish αB-crystallin is an ortholog of zebrafish αB2 and not αB1. However, several shared deletions between the catfish protein and zebrafish αB1 make this conclusion less definitive (Fig. 1). Surprisingly, the catfish αB-crystallin displays greater thermal stability than a porcine ortholog. In contrast, zebrafish αA-crystallin and αB1-crystallin are less thermostable than their mammalian orthologs [19], which is consistent with other studies that show reduced thermal stability of crystallin proteins from cooler-bodied ectothermic vertebrates [17,18].

Fish lenses contain lower concentrations of α-crystallins and higher concentrations of γ-crystallins than mammalian lens [17,35]. We quantified the relative amounts of the three α-crystallins in the zebrafish lens using 2D gel electrophoresis. On the basis of this analysis, zebrafish αB2 comprised only 0.16% of the adult lens total protein (Fig. 4). Zebrafish αA-crystallin and αB1-crystallin have nearly identical isoelectric points (5.8 and 5.7, respectively) and are similar in molecular mass; therefore, they migrated to an identical position on the gel and could not be differentiated. Together, the two proteins were far more prevalent than zebrafish αB2, making up 2.18% of the total lens protein. The total α-crystallin content of the zebrafish lens was far lower than the 30–40% typical of mammals, as has been previously reported for fish lenses. On the basis of a recent characterization of the catfish lens [34], the majority of this combined αA/αB1 spot on the 2D gel probably represents αA-crystallin. Additional studies will resolve αA-crystallin and αB1-crystallin and confirm the identity of modified and truncated products. The relatively high abundance and strong chaperonelike activity of αA-crystallin suggests a prominent role for this chaperone in the zebrafish lens, similar to that of mammalian αA-crystallin. In comparison, the low levels of αB2 in the zebrafish lens may indicate that its chaperone-like activity is less important in this tissue. However, the widespread expression of zebrafish αB2 suggests that it plays an important role similar to mammalian αB-crystallins in nonlens tissues. The physiological role of zebrafish αB1, with its lens-specific expression and decreased chaperone-like activity, still needs to be detailed.

This study shows that comparative analyses of non-mammalian species can provide novel insights into α-crystallin evolution and function. The two zebrafish αB-crystallins, which differ in chaperone-like activity and tissue expression, represent valuable models for investigating the functions of α-crystallins within and outside the vertebrate lens. In particular, the division of mammalian αB-crystallin functions between two separate zebrafish proteins can simplify the study of those functions. The zebrafish model also provides unique opportunities to use antisense gene knockdown and transgenesis techniques for *in vivo* analysis of gene function. Furthermore, comparative analysis of gene regulation using the two αB-crystallin genes makes the zebrafish an excellent model for examining the evolution of lens-specific expression. Mechanisms behind the evolution of tissue-specific expression are integral to understanding how lens crystallins became co-opted to produce transparent, refractive structures in the eye [36].

Experimental procedures

Cloning, sequencing and phylogenetic analysis

A cDNA library was constructed from adult zebrafish lens for the NEIBank project. Expressed sequence tag and bio-informatics analysis of almost 4000 clones revealed the presence of a second αB-crystallin gene transcript. Complete sequence was derived from expressed sequence tag reads of 30 clones, several of which contained the complete coding sequence, revealing major polymorphic sites and multiple polyadenylation sites. The accession numbers for all clones are listed in UniGene DR.32019 and can also be accessed through NEIBank (neibank.nei.nih.gov/index.shtml). The novel zebrafish αB-crystallin amino-acid sequence was deduced from the coding region and aligned with other vertebrate αB-crystallins using the algorithm CLUSTAL W [37]. A phylogenetic analysis of multiple α-crystallins and closely related sHSPs was performed using the program MEGA3 [38]. A neighbor-joining algorithm was used with Poisson correction, and the resulting tree was tested with 950 bootstrap replications. GenBank accession numbers for all sequences used in these analyses are indicated in the appropriate figures.

Semi-quantitative RT-PCR

Zebrafish were obtained from a local pet store, and total RNA was collected from brain, heart, lens, liver and skeletal muscle using the RNEasy kit (Qiagen, Valencia, CA, USA). All live animal procedures were approved by the appropriate institutional animal care committee. Total RNA from each tissue (6 $ng \cdot \mu L^{-1}$ concentrations) was subjected to RT-PCR using the Superscript One-Step system (Invitrogen, Carlsbad, CA, USA). Each sample was reverse-transcribed for 30 min at 50 °C, denatured at 94 °C for 2 min, and then amplified with the following primers, which were designed to span intron/exon boundaries to avoid amplification of genomic DNA: sense 5′-GCCGAC GTGATCTCCTCATT-3′; antisense 5′-CCAACAGGGA CACGGTATTT-3′. Cycle parameters were: 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. Aliquots from each reaction were collected at 20, 25 and 30 cycles. Preliminary reactions showed that 20 cycles was within the linear range of amplification for lens αB2-crystallin. The other tissues were still within linear range at 25 and 30 cycles. A parallel set of reactions was run without reverse transcriptase to further ensure that only RNA was amplified. A reaction containing water instead of total RNA was used as a negative control. Amplification products were excised from gels and sequenced to confirm their identity. Another set of reactions using tubulin-specific primers was performed to confirm that equal amounts of mRNA were used in each reaction. The tubulin reactions were performed for 30 cycles using the same parameters as above and the following primers: sense 5′-CTGTTGACTACGGAAAGAAGT-3′; antisense 5′-TATGTGGACGCTCTATGTCTA-3′.

2D gel electrophoresis

Approximately 10 μg adult zebrafish lens protein was applied to 7 cm immobilized pH gradient strips for the first dimension isoelectric focusing. The strips (pH 3–10, nonlinear; Amersham Biosciences, Piscataway, NJ, USA) were rehydrated in a solution of 7 M urea, 2 M thiourea, 4% CHAPS and 2.5 mg mL^{-1} dithiothreitol and focused for 16 000 $V \cdot h$ on the Protean IEF System (Bio-Rad, Hercules, CA, USA). The second dimension electrophoresis was on 16% Tris/glycine gels using the Novex Mini Cell apparatus (Invitrogen). Before the second dimension SDS/PAGE, the immobilized pH gradient strips were equilibrated at room temperature for 15 min in 50 mM Tris/6 M urea/30% glycerol/2% SDS (SDS equilibration buffer) containing 10 $mg \cdot mL^{-1}$ dithiothreitol followed by 15 min in SDS equilibration buffer containing 40 $mg \cdot mL^{-1}$ iodoacetamide. Gels were stained with GelCode Blue Stain (Pierce Biotechnology, Rockford, IL, USA) and scanned on a Personal Densitometer SI

(Molecular Dynamics). Progenesis image analysis software (Non-Linear Dynamics, Newcastle upon Tyne, UK) was used to quantify individual spots.

Production of recombinant protein and assays of chaperone-like activity

One full-length zebrafish αB2 clone was selected and used as template to amplify the coding sequence for cloning into the *Nde*I/*Xho*I sites of the pET20b(+) expression vector (Novagen, Madison, WI, USA). PCR primers used to amplify the coding sequence and incorporate appropriate restriction sites were: ZfaB2-5′, GCAGAAGAGGCCCAG ACTCCATATGGAC; ZfaB2-3′, CTCGAGAGTTGACGT TTAGCATCTTTAC. The sequence of the expression clone was verified. The expression construct was used to transform BL21(DE3) bacterial cells (Novagen). Protein expression, cell lysis and purification were performed essentially as described by Horwitz *et al*. [39] except for the following changes: Cell lysates were loaded on to a Mono Q Hi Trap column (Amersham) and eluted stepwise with 20 mM Tris/HCl, pH 8.5, with 0.1 M and 0.25 M NaCl. Fractions from the 0.25 M NaCl elution containing the recombinant crystallin were concentrated with Amicon centrifugal filters (30 kDa molecular mass cut-off; Millipore, Billerica, MA, USA) and passed through a 90 cm × 2.5 cm size-exclusion column containing Sephacryl S-200 High Resolution bedding material (Amersham) at a flow rate of 0.4 $mL \cdot min^{-1}$ and a temperature of 8 °C. Fractions containing purified α-crystallins were concentrated to ≈5 $mg \cdot mL^{-1}$ in Centricon YM-30 centrifugal concentrators (Millipore) and used in chaperone assays. A range of purified zebrafish αB2-crystallin concentrations was compared with known concentrations of human αB-crystallin on Coomassie stained polyacrylamide gels. The final concentrations of purified samples were quantified by densitometric analysis of these gels (Kodak 1D image analysis software; Eastman Kodak Co., Rochester, NY, USA).

Chaperone-like activities of purified zebrafish αB2-crystallin and human αB-crystallin were compared by measuring their ability to prevent the chemically induced aggregation of α-lactalbumin or lysozyme. α-Lactalbumin (L6010; Sigma, St Louis, MO, USA) was denatured with 20 mM dithiothreitol in a buffer containing 50 mM sodium phosphate/0.1 M NaCl, pH 6.75. Lysozyme (L6876; Sigma) was denatured with 1 mM Tris(2-carboxyethyl)phosphine hydrochloride in buffer containing 50 mM sodium phosphate and 0.1 M NaCl, pH 7.0. Absorbance due to light scattering produced in the reactions with or without the two α-crystallins was measured at 360 nm for 60–90 min at 27 °C and 37 °C. The abilities of purified zebrafish αB1-crystallin and αB2-crystallin and human αB-crystallin to prevent the aggregation of α-lactalbumin were also examined in triplicate over the temperature range 25–40 °C at 5 °C increments. All reactions were in a total of 500 μL using a 5-mm path length cuvette. The chaperone effectiveness of each crystallin was calculated as percentage protection against target protein aggregation. A one-way analysis of variance with Tukey-Kramer post test was used to determine whether the mean percentage protections of the three crystallins were significantly different at each temperature.

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Abbreviations

sHSP	small heat shock protein

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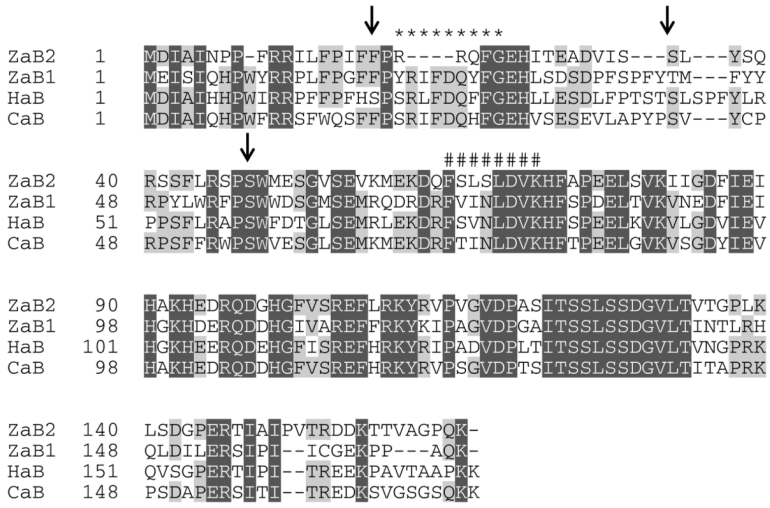

Fig. 1.

Amino-acid sequence alignment of several vertebrate αB-crystallins. Zebrafish αB2 (ZaB2; AAZ15808), zebrafish αB1 (ZaB1; AAD49096), human αB (HaB; AAB23453) and a catfish (*Clarius batrachus*) αB-crystallin (CaB; AAO24775) are shown. The alignment was produced using CLUSTAL W [37]. Grayed letters indicate amino acids shared between three of the sequences, and darkened letters represent amino acids identical between all four protein sequences. Dashes indicate gaps inserted within the sequence to optimize the alignments. Phosphorylation sites and a nine-amino-acid region (SRLFDQFFG in the human sequence) previously shown to contribute to structural stability are shown by arrows and asterisks, respectively. A possible eight-amino-acid chaperone-binding site (FSVNLDVK in the human sequence) is indicated above by number signs.

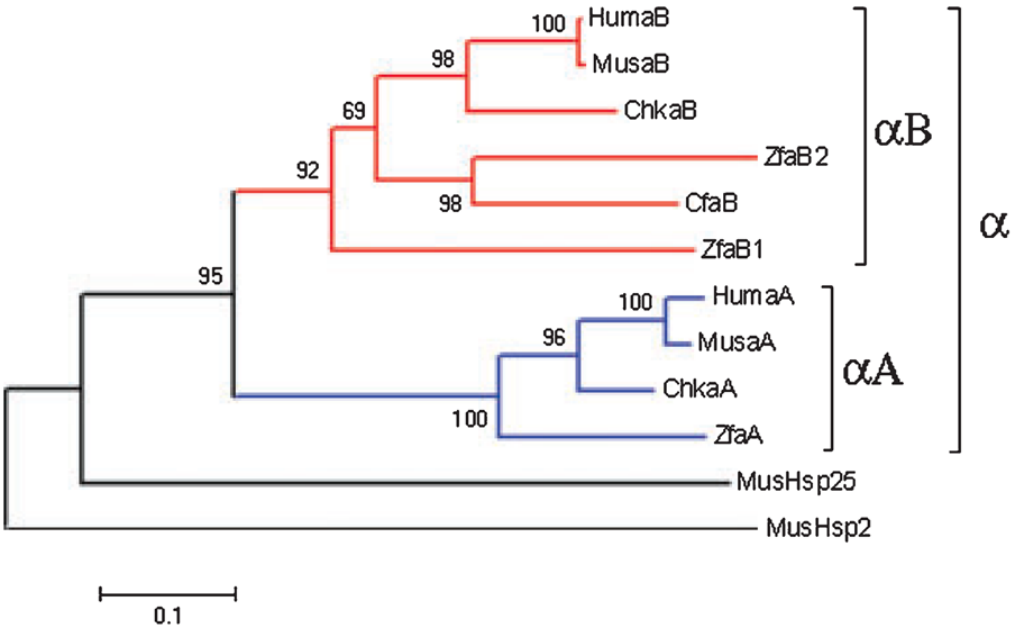

Fig. 2.

Phylogenetic tree of vertebrate α-crystallins and closely related sHSPs. The tree was calculated using MEGA3 with the neighbor-joining option and Poisson correction [38]. Numbers at the base of each node indicate bootstrap values out of 950 trees, and the scale bar indicates the number of substitutions per site. Amino-acid sequences included were human αB (HumaB; NP_001876), mouse αB (MusaB; AAH94033), chicken αB (ChkaB; Q05713), zebrafish αB2 (ZfaB2; AAZ15808), catfish αB (CfaB; AAO24775), zebrafish αB1 (ZfaB1, NP_571232), human αA (HumaA; AAB33370), mouse αA (MusaA; AAH92385), chicken αA (ChkaA; P02504), zebrafish αA (ZfaA; NP_694482), mouse HSP25 (MusHsp25; P14602) and mouse HspB2 (MusHsp2; Q99PR8).

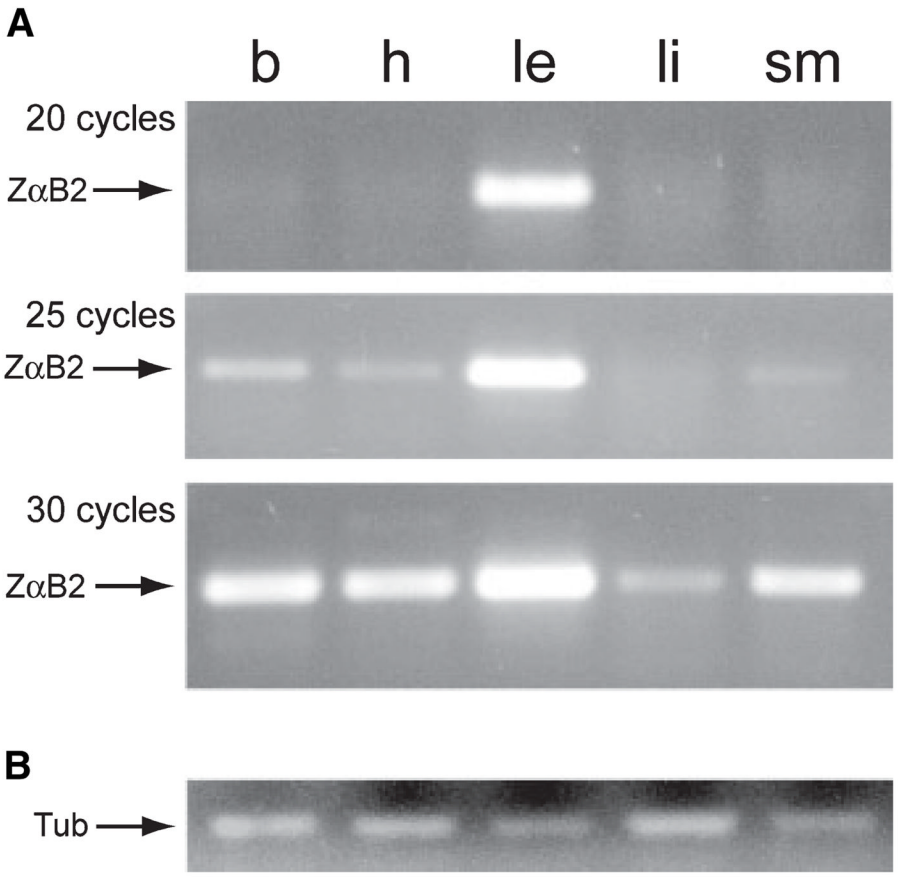

Fig. 3.

RT-PCR analysis of zebrafish αB2-crystallin expression. (A) Ethidium bromide-stained gels show amounts of amplified αB2-crystallin (ZαB2) from brain (b), heart (h), lens (le), liver (li) and skeletal muscle (sm) after the indicated number of cycles. (B) Ethidium bromide-stained gel showing amplification of tubulin (tub) as an internal control to ensure that equal amounts of mRNA were used from each tissue.

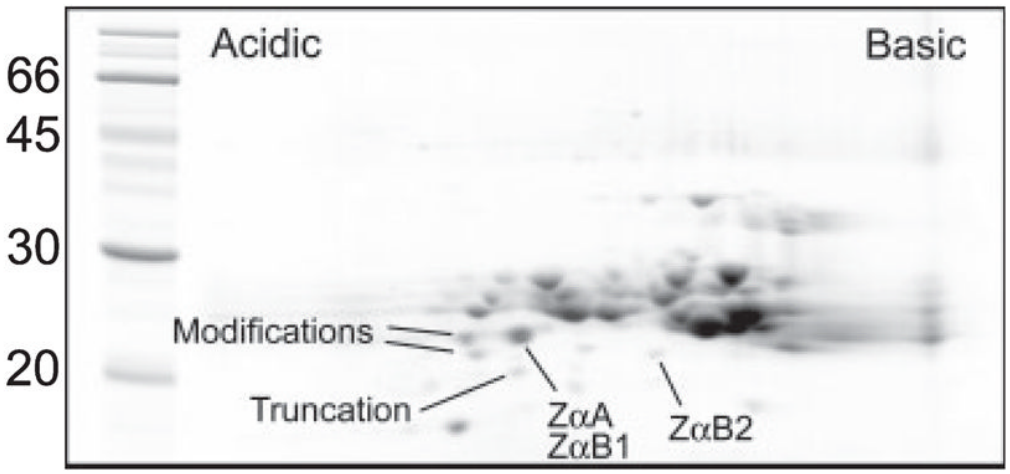

Fig. 4.

2D gel electrophoresis of zebrafish lens protein. The spots containing both zebrafish αA-crystallin and αB1-crystallin, zebrafish αB2-crystallin and modifications or truncations of α-crystallins are indicated. Molecular mass in kDa is shown on the left.

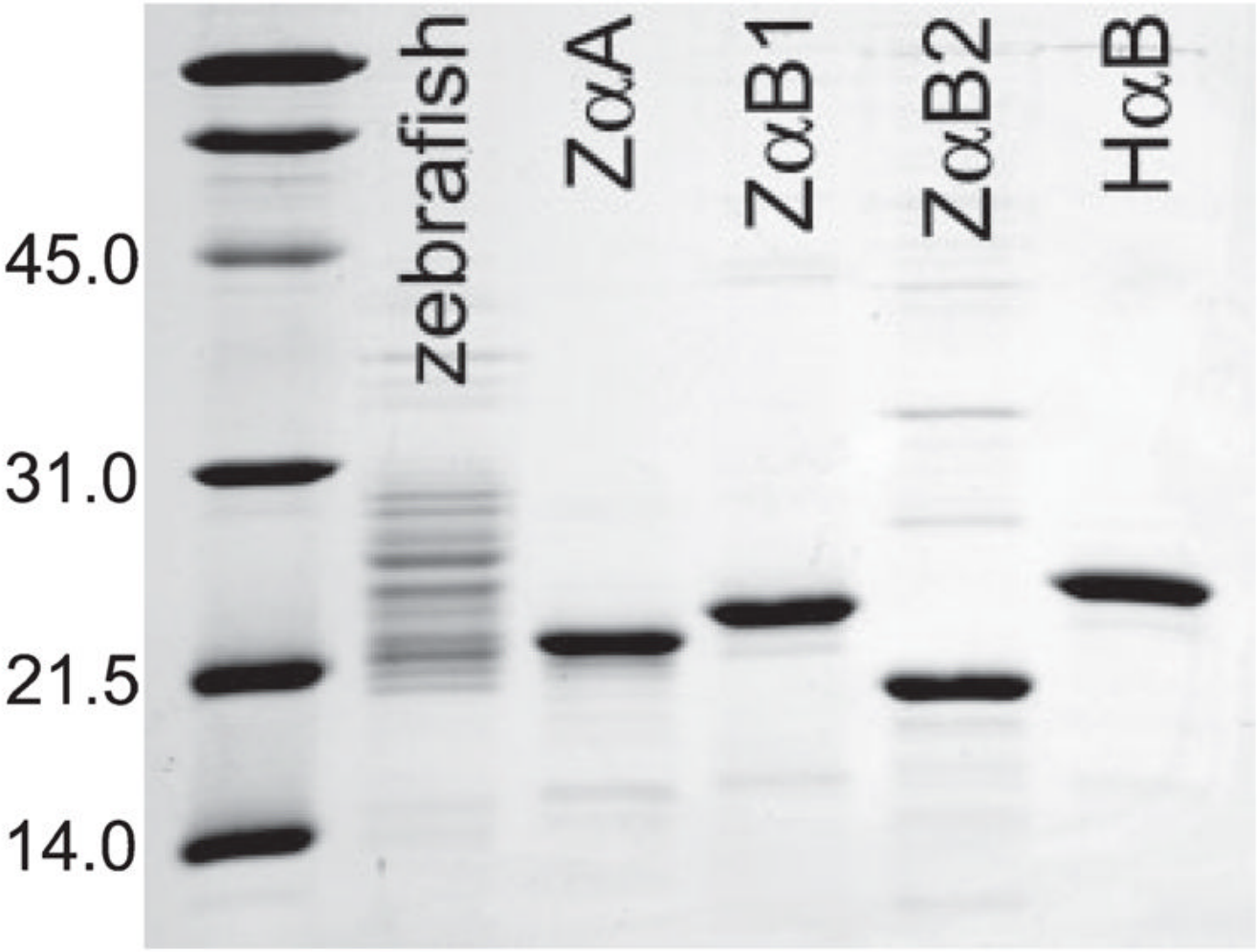

Fig. 5.
SDS/PAGE analysis of native zebrafish lens and various recombinant proteins. Four micrograms of total soluble zebrafish lens protein (zebrafish) and one microgram each of recombinant zebrafish αA-crystallin (ZαA), αB1-crystallin (ZαB1), αB2-crystallin (ZαB2) or human αB-crystallin (HαB) were electrophoresed in a 12.5% acrylamide gel. The molecular masses of standards (kDa) are indicated to the left.

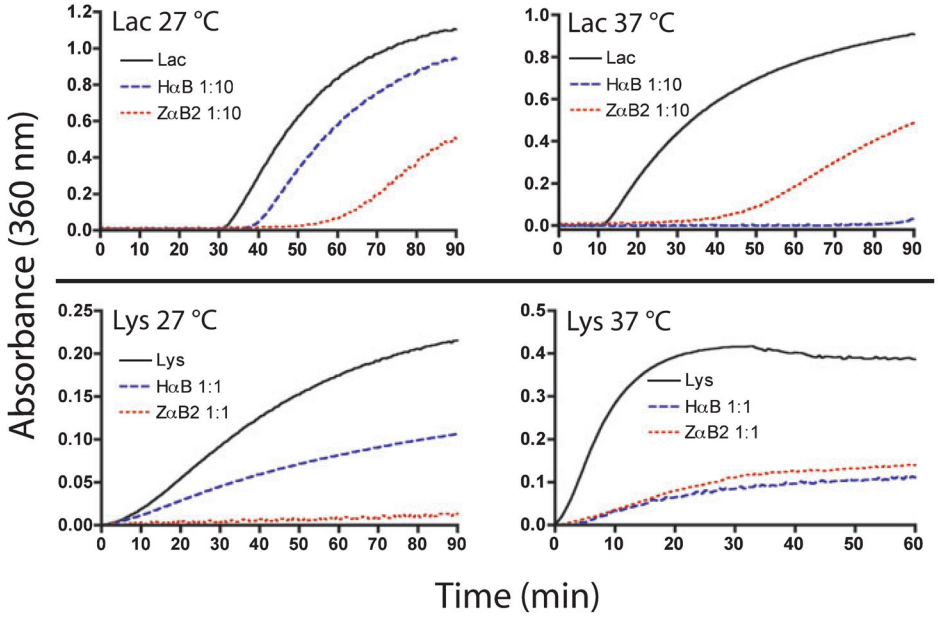

Fig. 6.

Chaperone-like activity of αB-crystallins at physiological temperatures. Assays were performed at 27 °C and 37 °C using α-lactalbumin (Lac; 0.6 mg·mL^{-1}) and lysozyme (Lys; 0.1 mg·mL^{-1}) as target proteins. These temperatures represent the physiological temperatures of the zebrafish and human, respectively. Curves indicate the aggregation of α-lactalbumin or lysozyme alone or with different ratios of added zebrafish αB2-crystallin (ZαB2) or human αB-crystallin (HαB). Ratios are shown as mass of crystallin/target protein. Lower absorbance indicates greater protection from aggregation provided by each of the crystallin proteins.

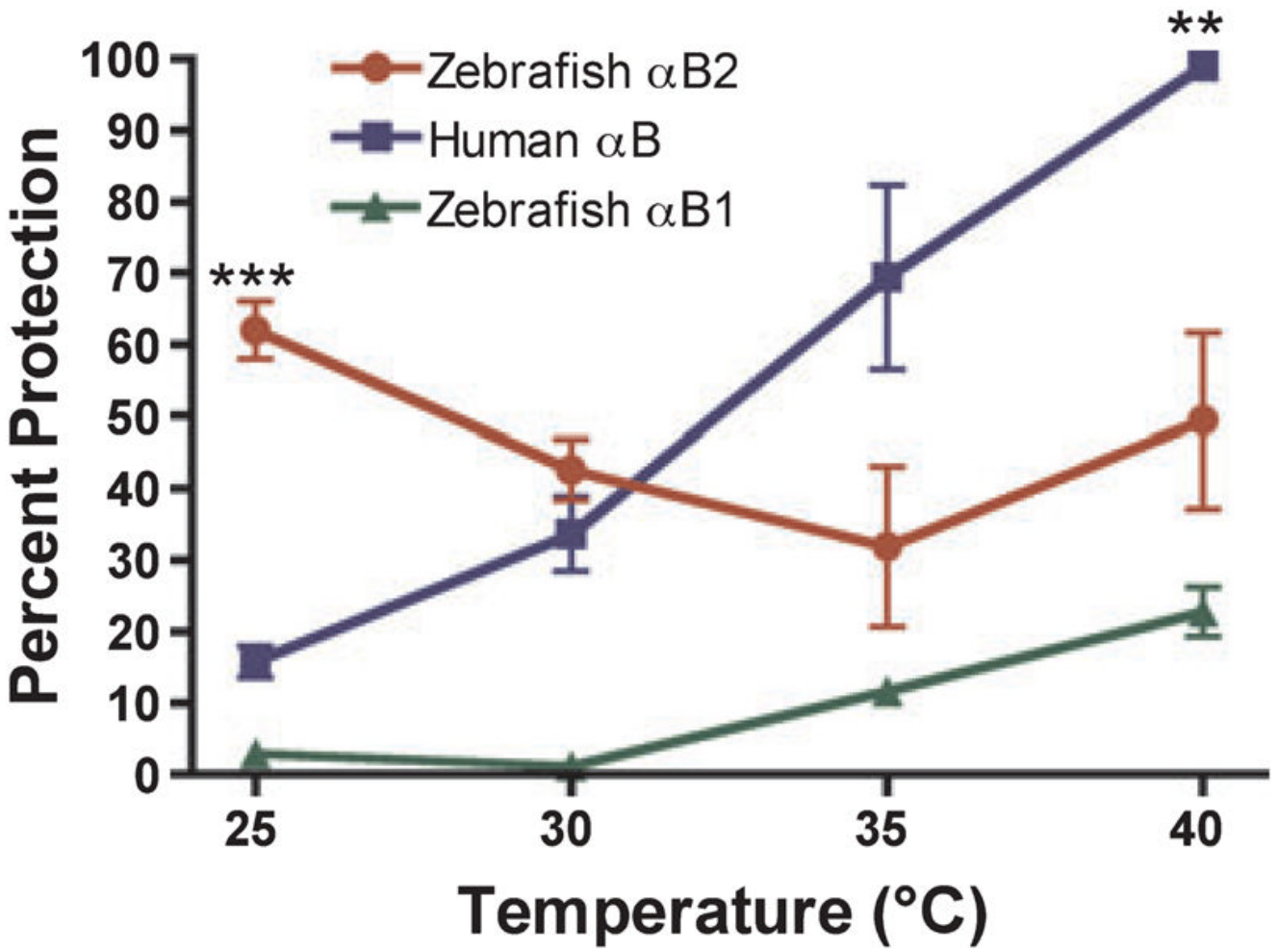

Fig. 7.

Temperature affects the ability of αB-crystallin to prevent α-lactalbumin aggregation. The ability of human αB-crystallin, zebrafish αB1-crystallin and zebrafish αB2-crystallin to prevent the aggregation of α-lactalbumin (0.6 mg·mL^{-1}) is shown at temperatures of 25–40 °C. Assays were conducted in triplicate at a mass ratio of 1: 10 crystallin to α-lactalbumin for 90 min. Data are means ± SEM (N = 3). Where error bars are not seen, they are contained within the symbol. Asterisks indicate statistically significant differences in mean percentage protection between zebrafish αB2-crystallin and human αB-crystallin (**P < 0.01, P < 0.001).