

Blind cavefish and heat shock protein chaperones: a novel role for hsp90 α in lens apoptosis

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ABSTRACT Lens apoptosis plays a central role in cavefish eye degeneration. Heat shock proteins (hsps) can regulate apoptosis; therefore, we examined the relationship between constitutive *hsp70* and *hsp90* expression and lens apoptosis. The model system is *Astyanax mexicanus*, a teleost species consisting of an eyed surface-dwelling (surface fish) form and numerous blind cave-dwelling (cavefish) forms. Optic primordia are formed in the cavefish embryo but they subsequently undergo lens apoptosis, arrest in development and degenerate. *Astyanax hsp90* and *hsp70* DNAs were isolated to use as probes to compare gene expression during surface fish and cavefish development. *Hsp90 β* , which encodes one of two hsp90 isoforms, was not expressed in the surface fish or cavefish lens, whereas *hsp70* was expressed in the lens of both forms, suggesting that neither is directly involved in lens apoptosis. In contrast, *hsp90 α* , the other hsp90 isoform, was expressed in the cavefish but not the surface fish lens. *Hsp90 α* expression peaked shortly before the beginning of lens apoptosis in three convergent cavefish populations, suggesting a close relationship with lens apoptosis. The absence of *hsp90 β* in the lens allowed us to use geldanamycin and radicicol, specific inhibitors of hsp90 chaperone function, to determine whether lens cell death requires *hsp90 α* expression. Both inhibitors blocked TUNEL labeling in the cavefish lens, suggesting that *hsp90 α* is required for apoptosis. In contrast to their effects on the lens, these inhibitors induced TUNEL labeling in the surface epidermis, presumably due to effects on hsp90 β function, implying that the two-hsp90 isoforms may have contrasting roles in cell survival. We conclude that hsp90 α plays a novel role in lens apoptosis and cavefish eye degeneration.

KEY WORDS: *cavefish, eye degeneration, lens apoptosis, hsp90 α , hsp90 β , hsp70*

Introduction

The heat shock proteins (hsps) are members of a small and highly conserved gene family encoding molecular chaperones: the cytoplasmic mediators of protein folding and metabolism. Three major hsp subgroups are distinguished by their relative molecular masses: hsp90 (~90kDa), hsp70 (~70kDa), and the low molecular weight hsps (16-47kDa). Although first identified as stress-inducible proteins, hsps were subsequently shown to have critical roles in basic cellular and developmental processes (Morimoto *et al.*, 1994; Richter and Buchner, 2001). Hsp70 is generally required for correct folding, assembly, intracellular targeting, and degradation of polypeptides and oligomeric proteins (Morimoto *et al.*, 1994). Under normal environmental conditions, hsp70 is strongly expressed in the lens of zebrafish (Blechinger *et al.*, 2002) and chick (Dash *et al.*, 1994) embryos. Hsp90 has more specialized chaperoning roles and most of its client proteins are members of key signal

transduction networks (Young *et al.*, 2001), such as the steroid hormone receptors (Picard *et al.*, 1990) and signaling kinases (Xu and Lindquist, 1993). Two hsp90 isoforms, hsp90 α and hsp90 β , are encoded by different genes and exhibit distinct developmental expression patterns in teleosts (Krone and Sass, 1994) and mammals (Gupta, 1995). In zebrafish, hsp90 α is involved in somite and axial muscle development (Lele *et al.*, 1999), whereas hsp90 β is preferentially expressed in the anterior epidermis and central nervous system (CNS), where its role is unknown (Krone and Sass, 1994).

Hsps are also implicated in evolutionary processes. Hsp70 can facilitate mutations through its involvement in a number of DNA sequence-alteration activities (Ogata *et al.*, 1993; Chow, 2000). Hsp90 is thought to capacitate phenotypic variation and morpho-

Abbreviations used in this paper: hsp, heat shock protein.

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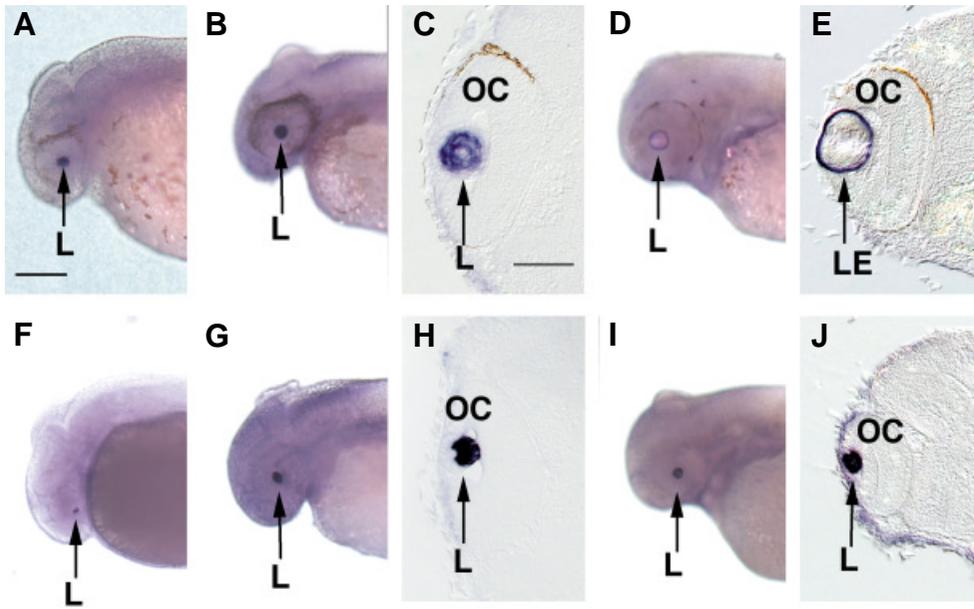


Fig. 1. Non-stress related *hsp70* expression in the lens during surface fish (A-E) and Pachón cavefish (F-J) development. Whole mount in situ hybridizations of 24 (A, F), 36 (B, G), and 48 (D, I) hpf embryos with developing optic regions viewed from a lateral side. Sections through in situ hybridized embryos showing the optic primordia at 36 (C, H) and 48 (E, J) hpf. L: lens. OC: optic cup. LE: lens epithelium. Scale bar in A is 100 μ m; magnification is the same in A, B, D, F, G, I. Scale bar in C is 50 μ m; magnification is the same in C, E, H, J.

logical evolution during periods of environmental flux by chaperoning developmental signaling components and masking phenotypic variation under normal environmental conditions (Rutherford and Lindquist, 1998; Queitsch *et al.*, 2002; Sollars *et al.*, 2003). Further understanding of the multiple roles of hsp proteins in development should contribute to our knowledge of how evolutionary diversity is generated.

We study the evolution of development in the teleost *Astyanax mexicanus*, a single species consisting of an eyed surface-dwelling form (surface fish) and several blind cave-dwelling (cavefish) forms (Jeffery, 2001). There are about 30 different populations of *A. mexicanus* cavefish with different genetic backgrounds and converged eyeless phenotypes (Dowling *et al.*, 2002). Although adult cavefish have degenerate eyes, eye primordia begin to develop in embryos but they later arrest and degenerate. The first point of divergence between surface fish and cavefish eye development occurs during lens differentiation. The fiber cells, which normally elongate and begin to express crystallin proteins, do not terminally differentiate and instead undergo apoptosis in the cavefish lens (Jeffery and Martasian, 1998; Yamamoto and Jeffery, 2000). In addition, the iris, cornea, and anterior chamber do not appear during cavefish eye development. The retina, which initially forms properly, does not differentiate the normal number of photoreceptor cells and gradually stops growing, although new retinal cells continue to be produced in the ciliary marginal zone (Strickler *et al.*, 2002).

The embryonic lens is an organizer of eye development (Beebe, 2000; Yamamoto and Jeffery, 2000; Kurita *et al.*, 2003). In blind cavefish, loss of this organizer activity through lens apoptosis results in eye degeneration. The central role of the lens in this process was demonstrated by lens transplantation experiments (Yamamoto and

Jeffery, 2000; Jeffery *et al.*, 2003). When a cavefish lens vesicle was placed into a surface fish optic cup lacking its own lens vesicle it underwent apoptosis and degeneration on schedule. Similar to the degenerate cavefish eye, the surface fish eye with the transplanted lens did not develop an iris, cornea, or anterior chamber, and retinal growth was inhibited. In the reciprocal experiment, a surface fish lens vesicle was transplanted into a cavefish optic cup after its own lens vesicle was removed. The transplanted surface fish lens developed normally in the cavefish host: an iris, cornea, and anterior chamber were formed, the retina increased in size, and photoreceptor cells differentiated. These results indicate that (1) apoptosis is an autonomous property of the cavefish lens, (2) the surface fish lens is capable of inducing the cavefish eye primordium to resist degeneration and develop normally, and (3) the cavefish lens has lost this inductive ability, presumably because of apoptosis. The cavefish system opens new

opportunities to study the role of apoptosis in generating an evolutionary change in development.

In addition to their other roles, hsps are also involved in apoptosis (Xanthoudakis and Nicholson, 2000). Hsp70 has been shown to protect cells from stress-induced apoptosis (Mosser *et al.*, 2000) by binding to the cell death mediator procaspase-9 and preventing its recruitment to the Apaf-1 apoptosome (Beere *et al.*, 2000). There is also evidence that hsp90 is involved in apoptosis (Pandy *et al.*, 2000; Wu *et al.*, 2002; Vanden Berghe *et al.*, 2003), although the mechanisms are still unclear. Because hsps play important roles in apoptosis, we have studied the expression patterns of *hsp70*, *hsp90 α* , and *hsp90 β* during cavefish lens development.

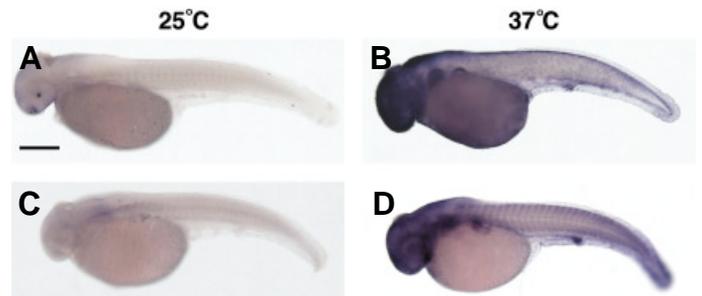


Fig. 2. Non-stress (A,C) and heat shock induced (B,D) patterns of *hsp70* (A,B) and *hsp90 α* (C,D) expression in 36 hpf Pachón cavefish embryos. Whole mount in situ hybridizations were developed for the same time in controls and heat shocked embryos. Hsp90 α is not seen in the lens or myotomes in (C) because the reaction was stopped early in order to avoid excess stain development after heat shock in (D). Scale bar in (A) is 200 μ m; magnification is the same in all frames.

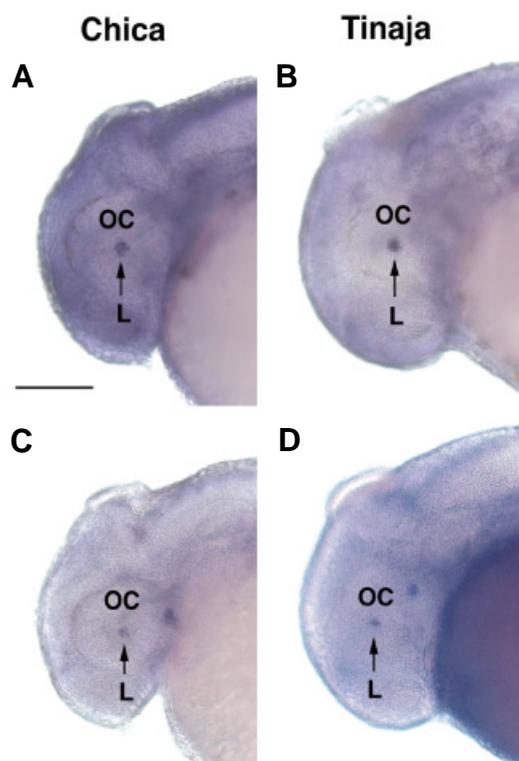


Fig. 3. Non-stress related *hsp70* (A,B) and *hsp90α* (C,D) expression in the lens of 36 hpf Chica (A,C) and Tinaja (B,D) cavefish embryos. Labels are the same as in Fig. 1. Scale bar in (A) is 100 μ m; magnification is the same in all frames.

We found that the patterns of *hsp70* and *hsp90β* expression in surface fish and cavefish embryos are essentially the same and do not suggest a direct role in lens apoptosis. However, we demonstrate here that *hsp90α* is specifically activated prior to lens apoptosis in several different cavefish populations and that pharmacological inhibition shows that *hsp90α* activation is necessary for inducing apoptosis in the cavefish lens. Our results suggest a novel role for *hsp90α* in lens apoptosis and eye degeneration in the blind cavefish.

Results

Hsp70 is expressed in the cavefish lens

To determine whether *hsp70* downregulation precedes lens apoptosis in cavefish, we cloned an *Astyanax hsp70* DNA fragment to generate an *in situ* hybridization probe. The *hsp70* DNA showed 86% and 95% conservation at the nucleotide level and amino acid levels respectively to zebrafish *hsp70* (Lele *et al.*, 1997).

In situ hybridization was conducted on surface fish and Pachón cavefish embryos between 16 and 72 hpf (Fig. 1). At normal growth temperature (25°C), *hsp70* expression was restricted to the developing lens (Figs. 1,2A), as described previously in zebrafish (Blechliger *et al.*, 2002). The pattern of *hsp70* expression was distinct from that of *hsp90α* and *hsp90β* (see Figs. 4,5). The constitutive *hsp70* mRNA level was greatly increased by a 1 hr 37°C heat shock and expression was expanded throughout surface fish and cavefish embryos (Fig. 2 A,B; data not shown), indicating that *hsp70* is heat inducible. In surface fish and cavefish

developing at normal temperature, *hsp70* mRNA was first detected at 24 hpf, shortly after the lens vesicle was formed from surface ectoderm (Fig. 1 A,F). *Hsp70* mRNA continued to be present in the surface fish and cavefish lens through 72 hpf (Fig. 1 B-E, G-J; data not shown), when lens apoptosis is occurring in cavefish (Jeffery and Martasian, 1998; Strickler and Jeffery, unpublished). In surface fish, *hsp70* expression was attenuated to the lens epithelium beginning between 36 and 48 hpf (Fig. 1 D,E), as crystallin-containing fiber cells begin to differentiate in the central core of the lens. At this time, *hsp70* mRNA was still present throughout the apoptotic cavefish lens (Fig. 1 I,J), which does not produce elongated lens fiber cells, although it does accumulate crystallin family mRNA and protein (Jeffery *et al.*, 2000). The latter was the only difference observed in *hsp70* expression during surface fish

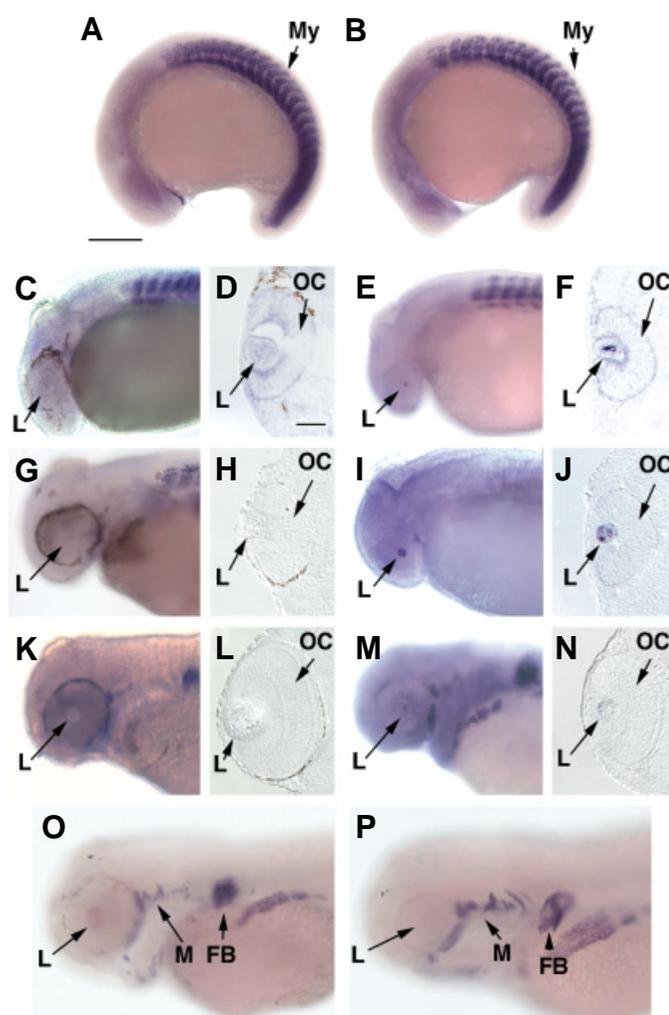


Fig. 4. Non-stress related *hsp90α* expression during surface fish (A, C, D, G, H, K, L, O) and Pachón cavefish (B, E, F, I, J, M, N, P) development. Whole mount *in situ* hybridizations of 18 somite (A, B), 24 hpf (C, E), 36 hpf (G, I), 48 hpf (K, M), and 72 hpf (O, P) embryos with developing optic regions viewed from a lateral side. Sections through the optic primordia of whole mount *in situ* hybridizations at 24 (D, F), 36 (H, J) and 48 (L, N) hpf. My, myotome; FB, fin bud; M, eye and pharyngeal muscles. Other labels are the same as in Fig. 1. Scale bar in (A) is 100 μ m; magnification is the same in (A-C, E, G, I, K, M, O, P). Scale bar in (D) is 30 μ m; magnification is the same in (D, F, H, J, L, N).

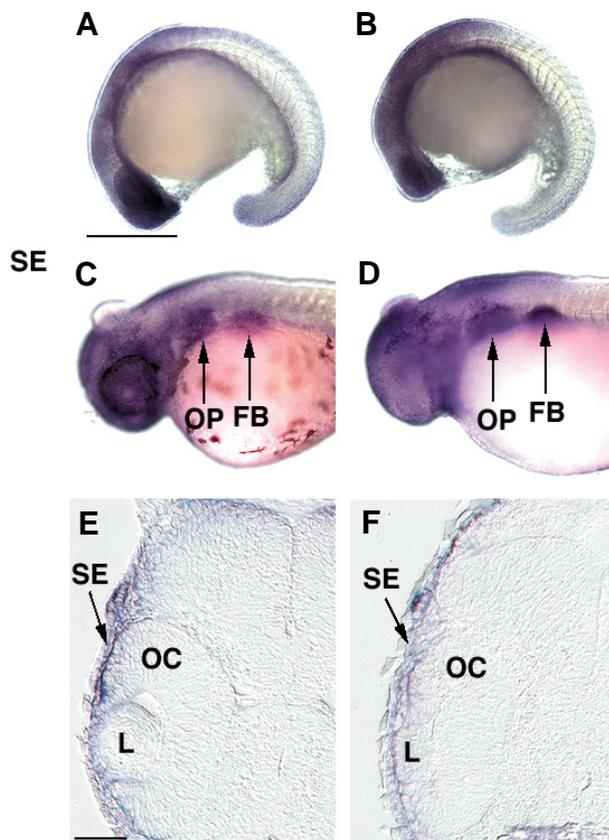


Fig. 5. Non-stress related *hsp90β* expression during surface fish (A, C, E) and Pachón (B, D, F) cavefish development. Whole mount *in situ* hybridization of 18 somite (A, B) and 36 hpf (C, D) embryos with developing optic regions viewed from a lateral side. (E, F) Sections through the optic primordia of whole mount *in situ* hybridizations at 36 hpf. OP, otic placode. Other labels are the same as in Figs. 1, 4. Scale bar in (A) is 100 μ m; magnification is the same in (A-D). Scale bar in (E) is 50 μ m; magnification is the same in (D, E).

and cavefish lens development. The results show that *hsp70* expression is stable during lens apoptosis in Pachón cavefish.

Other cavefish populations also exhibit lens apoptosis as a prelude to eye degeneration (Jeffery *et al.*, 2003). To assess whether *hsp70* is involved in lens apoptosis in some of these populations, the *hsp70* expression analysis was extended to Chica and Tinaja cavefish embryos. The results showed that *hsp70* mRNA was also expressed during the period of lens apoptosis in these divergent cavefish populations (Fig. 3 A, B). Therefore, we conclude that *hsp70* expression is not downregulated during cavefish lens development and that *hsp70* may not be directly involved in lens apoptosis.

***Hsp90α* expression is activated in the cavefish lens**

To determine if other hsp's may be involved in lens apoptosis, we compared *hsp90α* and *hsp90β* expression in surface fish and Pachón cavefish embryos. A full-length *hsp90α* *Astyanax* cDNA and an *hsp90β* DNA fragment were isolated. The *hsp90α* cDNA sequence showed 84% identity at the nucleotide level and 94% identity at the amino acid level to zebrafish *hsp90α* (Krone and Sass, 1994). The *hsp90β* DNA was 84% identical at the nucleotide

level and 95% identical at the amino acid level to zebrafish *hsp90β* (Krone and Sass, 1994).

In situ hybridizations were carried out between 16 and 72 hpf in surface fish and Pachón cavefish embryos (Fig. 4). Similar to the expression pattern seen in zebrafish embryos (Sass *et al.*, 1996; 1999), *hsp90α* mRNA was restricted to developing myotomes in early *Astyanax* embryos developing at 25°C (Fig. 4 A, B, C, E, G). Later, between 36 and 48 hpf, *hsp90α* mRNA decreased in the myotomes and was seen in developing muscle around the eye, pharyngeal muscle, pectoral fin buds, and a strip of tissue above the yolk mass that may represent developing gut muscle (Fig. 4 K, M, O, P). Expression in these tissues was the same in surface fish and cavefish embryos. A 37°C heat shock resulted in the intensification and expansion of *hsp90α* expression (Fig. 2 C, D), indicating that *hsp90α* is heat inducible in both forms of *Astyanax*. Remarkably, beginning at 24 hpf, *hsp90α* expression was detected in the cavefish lens vesicle (Fig. 4 E, F, I, J). *Hsp90α* expression in the cavefish lens peaked at 36 hpf and disappeared after 48 hpf (Fig. 4 I, J, M, N). The temporal pattern of *hsp90α* expression suggests that it presages lens apoptosis, which according to TUNEL analysis peaks at 48 hpf (Jeffery and Martasian, 1998; Strickler and Jeffery, unpublished). *Hsp90α* mRNA was not detectable during surface fish lens development (Fig. 4 C, D, G, H, K, L, O). As in zebrafish (Lele *et al.*, 1999), the pattern of *hsp90β* expression differed entirely from that of *Hsp90α*. *In situ* hybridization showed *hsp90β* transcripts in the anterior epidermis and CNS, otic placodes, and pectoral fin buds, but not in the lens, and there were no differences in surface fish and cavefish (Fig. 5). The results show that *hsp90α*, but not *hsp90β*, is specifically activated prior to the beginning of apoptosis in the Pachón cavefish lens.

When *in situ* hybridization was extended to other cavefish populations, we found that *hsp90α* expression also peaked before apoptosis in the lens vesicles of Chica and Tinaja cavefish embryos (Fig. 3 C, D). The results suggest that *Hsp90α* may be involved in lens apoptosis in diverse cavefish populations.

***Hsp90α* inhibition shows a requirement for lens apoptosis**

Hsp90α activation in the cavefish lens could be the cause or effect of apoptosis. We initially attempted to study the role of *hsp90α* in lens apoptosis by injecting antisense *hsp90α* morpholinos into cavefish eggs and *hsp90α* mRNA into surface fish eggs. However, morpholino injection at several different concentrations resulted in embryonic lethality prior to the formation of a morphologically distinguishable eye primordium, probably because of the major role of this molecular chaperone in early somite development (Lele *et al.*, 1999). Furthermore, injection of *hsp90α* mRNA produced no recognizable phenotype, perhaps due to the difficulty of

TABLE 1

SUPPRESSION OF LENS APOPTOSIS IN CAVEFISH EMBRYOS BY HSP90 INHIBITION

Treatment	Number of lenses				Total lenses (N)	
	Number of TUNEL stained nuclei in section	0	1-5	6-10		10+
Control		0	1	3	10	14
Geldanamycin		5	11	7	2	25
Radicicol		4	6	4	0	14

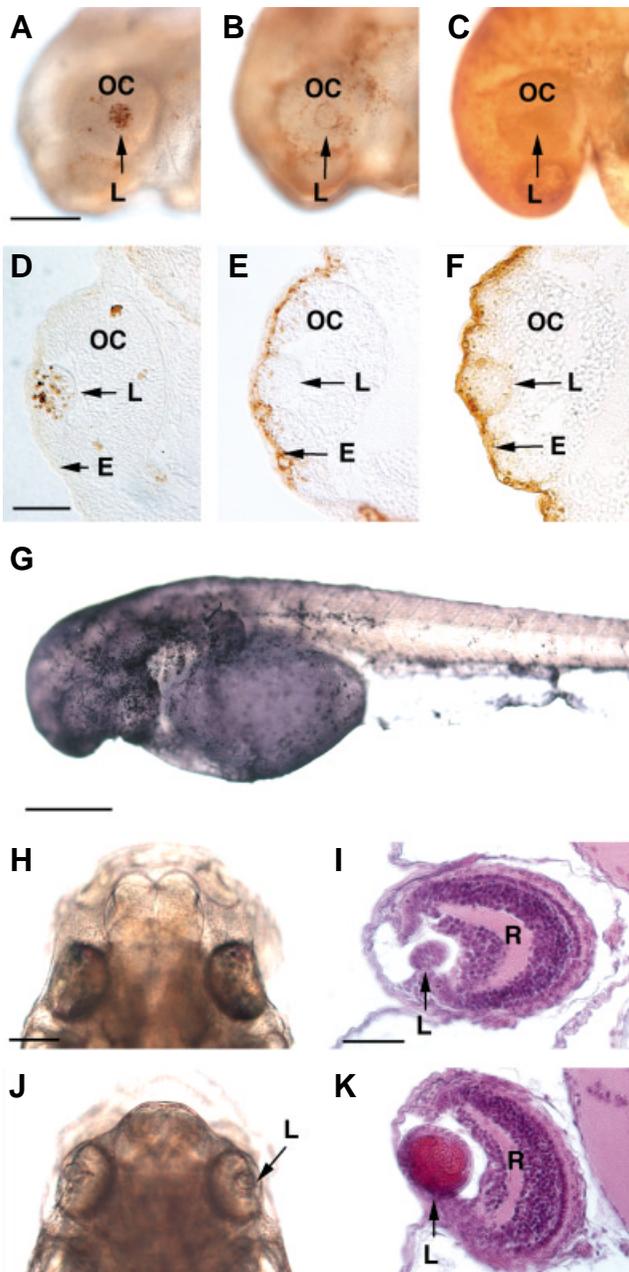


Fig. 6. Effects of pharmacological inhibition of hsp90 on cavefish lens apoptosis. (A-F) TUNEL stained whole mounts showing lateral views of the eye (A-C) and corresponding sections through the lens and optic cup (D-F) in control (A,D), 20 μ g geldanamycin-treated (B,E), and radicicol-treated (C,F) cavefish embryos at 48 hpf. After drug treatment, apoptotic cells were less prevalent in the lens but appeared in the surface ectoderm (E,F). (G) A 56 hpf cavefish embryo treated with 10 μ g/ml geldanamycin showing extensive TUNEL labeling in anterior epidermis. (H-K) Rescue of lens growth and differentiation in cavefish treated with radicicol. (H,I) Controls. (J,K) Radicicol treatment. (H,J) Dorsal views of the optic regions at 6 dpf showing increased lens size after radicicol treatment (K). (I,K) Sections through the lens and optic cup showing eosin-stained central core indicative of lens fiber differentiation after radicicol treatment (K). Controls do not show lens core development (I). E, epidermis. R, Retina. Other labels are the same as in Fig. 1. Scale bar in (A) is 100 μ m; magnification is the same in (A-C). Scale bar in (D) is 50 μ m. Scale bar in (G) is 200 μ m; magnification is the same in (G,I). Scale bar in (H) is 60 μ m; magnification is the same in (H,J). Scale bar in (I) is 50 μ m; magnification is the same in (I,K).

development. Subsequent development was followed in some of the radicicol treated embryos (Fig. 6 H-K). At 6 days post-fertilization (dpf), the size of the lens was markedly increased relative to controls and fiber cell differentiation was rescued in some of these embryos, as determined by the formation of a crystalline lens core (Fig. 6K), a feature not seen in untreated controls (Fig. 6I). The results indicate that inhibition of hsp90 function can prevent lens apoptosis, suggesting that it has a causal role in this process.

In striking contrast to their inhibitory effects on lens apoptosis, geldanamycin and radicicol induced apoptosis in the surface epidermis (Fig. 6 E-G). Since these drugs inhibit both hsp90 α and hsp90 β function (Lele *et al.*, 1999), and the latter is expressed in surface epidermis (Fig. 5), we conclude that hsp90 β normally protects these cells from apoptosis during early development. The results suggest that different hsp90 isoforms may have contrasting roles in promoting or suppressing apoptosis.

Discussion

We have studied *hsp70* and *hsp90* expression during lens apoptosis in the cavefish *Astyanax*. Our results suggest that *hsp70* and *hsp90 β* are not directly responsible for lens apoptosis. However, we demonstrate that *hsp90 α* , which is expressed in the cavefish lens immediately prior to the beginning of apoptosis, is required for lens cell death. Our studies suggest a novel role for the molecular chaperone hsp90 α in the evolution of lens apoptosis and the eyeless phenotype in blind cavefish.

The incentive for considering hsp70 as a regulator of cavefish lens apoptosis was based on two previous findings. First, under non-stress conditions, *hsp70* is known to be expressed in the lens during early chick and zebrafish development (Dash *et al.*, 1996; Blechinger *et al.*, 2000). The role of *hsp70* expression in the developing lens is unknown. Second, hsp70 has been shown to act as a survival factor, protecting mammalian cells from stress-induced apoptosis by direct physical interaction with components of the cell death machinery (Beere *et al.*, 2000). Thus, we hypothesized that hsp70 may protect developing lens cells from apoptosis during the critical steps of lens morphogenesis and differentiation and that hsp70 loss could trigger apoptosis in the cavefish lens. As in zebrafish (Blechinger *et al.*, 2002), we found that *hsp70* is

partitioning mRNA to the lens in sufficient concentration to elicit an effect. We next examined the effects of geldanamycin and radicicol on lens apoptosis. These structurally different compounds specifically inhibit hsp90 chaperone function by preventing the ATP-dependent release of client proteins (Whitesell *et al.*, 1994; Roe *et al.*, 1999).

Cavefish embryos were pre-treated with geldanamycin or radicicol beginning at 24 hpf and the drug treated and control embryos were fixed and subjected to TUNEL assay at 48 hpf, during the peak of lens apoptosis. The results showed that lens apoptosis was greatly reduced relative to controls when embryos were treated with either drug (Fig. 6 A-F; Table 1). In drug-treated embryos, however, *hsp90 α* expression still peaked at 36 hpf (data not shown), indicating that the pharmacological effects cannot be attributed to a delay in the normal program of cavefish lens

strongly expressed in the developing *Astyanax* lens. There are no striking changes in *hsp70* expression, however, either between surface fish and cavefish or in the developing cavefish lens before or during apoptosis. Three different cavefish populations failed to show changes in *hsp70* expression associated with lens apoptosis. Thus, blind cavefish do not appear to activate the lens cell death pathway by down-regulating *hsp70*. Our results suggest that *hsp70* is not directly involved in cavefish lens apoptosis, although they do not discount the possibility that this molecular chaperone may function as a survival factor during lens development. If *hsp70* does have a survival role in the lens our results imply that it must somehow be negated by the activity of *hsp90 α* .

The analysis of *hsp90* expression during *Astyanax* development was instigated by mounting evidence that *hsp90*, which has a much more specific range of chaperone activities and client proteins than *hsp70*, may be a regulator of apoptosis (Xanthoudakis and Nicholson, 2000). We report here that *hsp90 α* is activated in the cavefish lens during embryonic development. The lens is the only tissue, other than developing somites and muscle cells, where we observed reproducible *hsp90 α* expression during early cavefish development. Moreover *hsp90 α* is the first gene we have discovered that is exclusively expressed in the cavefish lens. Other lens specific genes, such as those encoding β - and gamma crystallins, are expressed in both surface fish and cavefish embryos (Jeffery *et al.*, 2000). Expression of *hsp90 β* , the other *hsp90* isoform, could not be detected in the *Astyanax* lens, although it was expressed in the anterior epidermis, CNS, and otic placodes, as previously reported in zebrafish (Krone and Sass, 1994; Lele *et al.*, 1999). Our results show that the two *hsp90* isoforms have distinct expression patterns during *Astyanax* surface fish and cavefish development.

Several lines of evidence support the hypothesis that *hsp90 α* activation is responsible for apoptosis in the cavefish lens. First, *hsp90 α* expression peaks during a narrow window of time immediately prior to the beginning of nuclear DNA fragmentation as detected by TUNEL in dying cavefish lens cells. Second, *hsp90 α* expression occurs during this critical time interval in three divergent cavefish populations (Dowling *et al.*, 2002). This evolutionary convergence in *hsp90 α* activation suggests a close relationship with eye degeneration. Third, inhibition of *hsp90* function by geldanamycin or radicicol can block lens apoptosis. Although the two drugs interfere with the chaperone activity of both *hsp90* isoforms (Lele *et al.*, 1999), the absence of *hsp90 β* expression in the lens points to *hsp90 α* as the critical apoptotic regulator. These studies rule out the possibility that *hsp90 α* , acting as a survival factor, is expressed in the cavefish lens in a final but unsuccessful bid to prevent cell death. If this were the case, apoptosis would not be expected to decrease following *hsp90* inhibition.

Most previous work has implicated *hsp90* as a protector rather than an inducer of apoptosis. For example, stabilization of RIP-1 kinase, an anti-apoptotic protein, is dependent on binding with *hsp90* (Lewis *et al.*, 2000; Vanden Berghe *et al.*, 2003). In the absence of *hsp90*, RIP-1 is degraded and cells are sensitized to apoptosis. The only other indication of *hsp90 α* pro-apoptotic activity is a recent report suggesting that it promotes nicotine-induced apoptosis in human cultured cells (Wu *et al.*, 2002). The present results implicating *hsp90 α* in lens apoptosis suggest that this molecular chaperone may be able to promote or prevent apoptosis in different tissues and at different times in development, perhaps depending on the client protein(s) being chaperoned. It is also clear that in some situations

hsp90 α is neutral with regard to apoptosis. A case in point is that although *hsp90 α* is strongly expressed in teleost myotomes and other muscle cells (the present study; Sass *et al.*, 1996), these tissues do not show extensive apoptosis during *Astyanax* development (Hooven and Jeffery, unpublished).

It is conceivable that *hsp90 α* and *hsp90 β* have contrasting roles in cell death: the former promoting and the latter preventing apoptosis. This possibility is supported by our results showing geldanamycin and radicicol inhibition of apoptosis in the lens, where *hsp90 α* is expressed, but induction of apoptosis in the surface epidermis, where *hsp90 β* is expressed. Interestingly, most previous pharmacological and biochemical studies, including those implicating *hsp90* as a survival factor, have not distinguished between the individual effects of its two isoforms.

It remains unclear as to exactly how *hsp90 α* may initiate apoptosis in the cavefish lens. Two alternative models are proposed here, assuming that the hsps in question vary in the same fashion as their mRNAs. The first possibility is that *hsp90 α* may initiate lens apoptosis directly through binding to and activating a component of the cell death machinery, such as pro-caspase 3. In this light, it must be considered that lens fiber cells undergo a unique process resembling apoptosis during normal differentiation, which is required for the elimination of organelles and optical clarification (Lang, 1997; Wride, 2000). Accordingly, *bcl-2* and caspase family proteins are normally expressed in the chick lens, and *bcl-2* overexpression and caspase inhibitors can block fiber cell differentiation (Wride *et al.*, 1999). Therefore, *hsp90 α* activation could function somewhere in the cell death pathway downstream of caspase-3 to drive the lens apoptotic process to completion. Further studies of the factors activated downstream of *hsp90 α* could provide insight into how it promotes apoptosis.

The alternative possibility is that *hsp90 α* mediates lens apoptosis indirectly by interfering with the normal protective function of an anti-apoptotic factor or hsp. *Hsp70*, which suppresses caspase activation by targeting Apaf-1 and the anti-apoptotic protein Bag-1, would be a prime candidate for this interaction, although there are others, including *hsp27*, which also has a major role in cell survival (Xanthoudakis and Nicholson, 2000). During activation of the estrogen receptor complex, *hsp70* binding is followed by an interaction with dimeric *hsp90* (Smith *et al.*, 1992; Chen and Smith, 1998; Hutchison *et al.*, 1994), an indication that multimeric protein complexes containing both *hsp70* and *hsp90* are present in some cells. In order to distinguish between the direct and indirect models for *hsp90 α* function, it will be necessary to investigate *hsp90 α* interaction with other proteins in the developing cavefish lens.

In addition to functioning as a molecular chaperone during normal development and cellular stress, *hsp90* appears to be involved in evolutionary processes such as the generation of novel phenotypes (Rutherford and Lindquist, 1998; Queitsch *et al.*, 2002; Sollars *et al.*, 2003). We have shown here that *hsp90 α* has an important role in regressive evolution of the eye in blind cavefish.

Materials and Methods

Animals

Astyanax surface fish were collected at Balmorhea State Park, Texas and cavefish were collected from La Cueva de El Pachón (Pachón cavefish) in Tamulipas, Mexico and from La Cueva Chica (Chica cavefish) and El Sótano de la Tinaja (Tinaja cavefish) in San Luis Potosí, Mexico. Laboratory raised stocks were maintained in a custom built recycling

aquarium system (Marine Biotechnology Inc, Beverly, MA). Embryos were obtained by natural spawning and cultured in recycled tank water (RTW) at 25°C as described previously (Jeffery *et al.*, 2000). Under these conditions, gastrulation occurs at about 4-6 hours postfertilization (hpf), the tailbud stage at 10-11 hpf, the 18 somite stage at 16 hpf, lens vesicle formation at 22 hpf, hatching at 24 hpf, and lens apoptosis (in cavefish) begins at 36 hpf.

Isolation of hsp90 α , hsp90 β and hsp70 cDNA

Total RNA extracted from 18 somite surface fish was used to generate a Uni-ZAP XR cDNA library (Stratagene, La Jolla, CA). RT PCR was performed using the PCR Master Kit (Roche, Indianapolis, IN), cDNA library template, and the following degenerate oligonucleotide primers:

hsp90 α 5'-TBMGRGARCTSATCTCCAAC-3' (forward) and

5'-RGWGATGTCATCDGGGTTTC-3' (reverse);

hsp90 β 5'-TGATTGGGCAGTTTGGTGT-3' (forward) and

5'-GTCGTTGGTCAGGCTYTTGT-3' (reverse);

hsp70 5'-ACGCAGCHAAGAACCAGGTG-3' (forward) and

5'-CGTGGGYTCGTTGATRAT-3' (reverse). After 35 PCR cycles at an

annealing temperature of 55°C, the reactions yielded fragments of the expected sizes: 516 bp (*hsp90 α*), 580 bp (*hsp90 β*), and 375 bp (*hsp70*). These fragments were gel-extracted, ligated into the PCRScript vector (Stratagene), and sequenced. Blast analyses (Altschul *et al.*, 1997) of all three nucleotide and translated amino acid sequences revealed significant and unmatched homology to corresponding zebrafish DNA fragments.

The full-length *hsp90 α* coding sequence was obtained by screening the cDNA library with a digoxigenin-labeled DNA probe generated from the 516 bp PCR fragment of *Astyanax Hsp90 α* . Two rounds of screening were conducted using Stratagene supplied procedures. After *in vivo* excision of the pBluescript SK phagemid (Stratagene), DNA sequence analysis of seven putative *Hsp90 α* clones revealed six containing the same complete 2170 bp coding region, which has been deposited in GenBank (AY222612).

In situ hybridization

The DIG RNA Labeling Kit (Roche) was used to generate antisense and sense digoxigenin-UTP-labeled RNA probes from the *hsp90 α* , *hsp90 β* , and *hsp70* cloned fragments. At the appropriate stage, embryos were fixed in 4% paraformaldehyde (PFA)/PBS at 4°C overnight. They were then dehydrated in methanol and stored at -20°C. Prior to *in situ* hybridization, the fixed embryos were rehydrated in PBST (1X PBS, 0.1% Tween 20), hybridized at 65°C overnight, and stained following the procedures described by Püschel *et al.* (1992). After staining, embryos were photographed, embedded in polyester wax, and sectioned at 8 μ m.

Heat shock

At 36 hpf, 20-25 surface fish or cavefish embryos were transferred to 60 mm x 15 mm plastic petri dishes containing RTW, and the dishes were floated on the surface of a 37°C water bath. After 1 hr at 37°C, the heat shocked embryos, as well as 25°C control embryos from the same spawn, were processed for *in situ* hybridization.

TUNEL assay

Fixed and dehydrated embryos were rehydrated in PBT (1x PBS, 0.1% Triton X-100). Embryos were then washed twice for 5 min with PBT, treated with Proteinase K (10 μ g/ml in PBT) for 45 min, washed twice for 5 min with PBT, post-fixed in 4% PFA/PBT for 20 min, and washed twice with PBT for 5 min. These steps were carried out at room temperature. The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche) with detection of fragmented DNA by peroxidase and Sigma Fast, 3,3'-diaminobenzidine (Sigma Chemicals, St. Louis, MO) according to instructions supplied by the manufacturers. Stained embryos were photographed, embedded in polyester wax, and sectioned at 8 μ m.

Pharmacological hsp90 inhibition

At 24 hpf, groups of 25-30 cavefish embryos were transferred from RTW to 5 ml of the following solutions: 10 or 20 μ g/ml geldanamycin (Sigma) in

1% DMSO/RTW, 2.5 μ M radicicol (Sigma) in 1% DMSO/RTW, 1 μ M radicicol in 1% DMSO/RTW, and 1% DMSO/RTW (negative control). At 48 hpf all embryos treated with geldanamycin and 2.5 μ M radicicol, and half of the 1% DMSO control embryos, were fixed in 4% PFA/PBS and processed for *in situ* hybridization or TUNEL. The 1 μ M radicicol treated and remaining 1% DMSO control embryos were allowed to continue development until 6 d post-fertilization (dpf), then were fixed in Bouin's solution overnight at 4°C, dehydrated in ethanol, embedded in Paraplast, and sectioned at 8 μ m. Sections were stained with eosin and hematoxylin following standard procedures.

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