Expression of the chaperonin 10 gene during zebrafish development

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Abstract We have isolated a cDNA encoding chaperonin 10 (cpn10) from the zebrafish. Using northern, western, and in situ hybridization analysis, we observed that the cpn10 gene is expressed uniformly and ubiquitously throughout embryonic development of the zebrafish. Upregulation of cpn10 expression was observed following exposure of zebrafish embryos to a heat shock of 1 hour at 37°C compared to control embryos raised at 27°C. The extracellular form of Cpn10 called early pregnancy factor (EPF), found in the serum of pregnant mammals, was not detected in the serum of either male or female zebrafish. These expression studies suggest that Cpn10 plays a general role in zebrafish development as well as being consistent with the hypothesis that EPF is involved in the embryo implantation process in mammals.

INTRODUCTION

The proper folding and assembly of proteins within a cell is often assisted by a group of proteins called chaperones. Two of the most widely studied chaperones from *Escherichia coli*, GroEL and GroES, are members of the chaperonin family (Hemmingsen et al 1988) and are involved in protein folding in this and other prokaryotes. Highly conserved homologues of GroEL and GroES, termed Cpn60 (Hsp60) and Cpn10 (Hsp10), have been found in eukaryotes and are involved in protein folding in the mitochondria and plastids of plants (Lubben et al 1990; Hartman et al 1992).

In eukaryotes, Cpn10 has also been suggested to have a number of nonchaperone functions in addition to the important role in the folding and assembly of proteins within a cell. Early pregnancy factor (EPF), a protein found in the serum of pregnant mammals, has been identified as an extracellular homologue of Cpn10 (Cavanagh and Morton 1994). EPF is first detected in the maternal serum within 24 hours of fertilization and persists until midgestation (Morton et al 1987). Studies in mammals demonstrated that EPF is released into the serum from the ovary in response to the presence of a viable embryo. It is thought to have roles in maintaining embryonic viability by the fact that passive immunization of mice against EPF leads to failure to maintain pregnancy (Athanasas-Platsis et al 1989, 1991). Two hypotheses have been suggested for Cpn10 in the maintenance and development of a viable mammalian embryo. First, EPF may play a role in preventing an immunogenic reaction against the developing embryo since it has been shown to induce the production of factors that suppress immunological responses (Rolfe et al 1988). Second, the fact that EPF is produced by actively dividing tumor cells and that antibodies to EPF perturb tumor cell growth suggest that it may act as a growth factor (Quinn et al 1990). The zebrafish is an excellent developmental model system because the embryos can be readily obtained, are optically clear, and permit easy visualization of developmental events. Furthermore, the available genetic and molecular tools permit detailed molecular studies to be undertaken. Since fish are ancestral vertebrates, the zebrafish can also be used to address questions regarding ancestral function.

Our laboratory has been involved in studying the roles of a number of heat shock proteins, including Hsp47, Hsp70, and Hsp90α, in the development of the zebrafish embryo (Sass et al 1996; Lele and Krone 1997; Lele et al 1997; Sass and Krone 1997; Sass et al 1999; Lele et al 1999). In order to investigate the role of chaperonin 10 in embryonic development, we have cloned a zebrafish *cpn10* cDNA and analyzed the expression of this gene during the development of the zebrafish embryo under normal and stress conditions (heat shock). To determine
if the ovary of gravid female zebrafish produces EPF as the ovary does in mammals, serum was collected from adult male and adult gravid female zebrafish and analyzed for the presence of Cpn10 protein. We show that cpn10 is expressed uniformly and ubiquitously throughout the development of the zebrafish embryo and that cpn10 expression is heat inducible. Unlike the situation in mammals, we did not observe Cpn10 protein in either male or female zebrafish serum.

MATERIALS AND METHODS

Animals

Embryos were obtained from zebrafish purchased from a local supplier or spawned and raised in house. Fish were maintained using standard methods (Westerfield 1995). Embryos were maintained at 27°C and staged according to hours postfertilization (Kimmel et al 1995). Heat shock experiments involved placing embryos in a 35-mm petri dish that was then floated in a 37°C water bath. Control embryos remained at 27°C.

Degenerate RT-PCR

Total mRNA was isolated from 24-hour zebrafish embryos subjected to heat shock at 37°C for 1 hour. First strand synthesis was performed at 37°C employing 1 µg of mRNA, 50 pmol of a 6-mer poly-A primer, and 200 U of Moloney leukemia virus reverse transcriptase (Gibco BRL) according to manufacturer’s instructions. cDNA derived from 100 ng of mRNA was used for PCR reactions containing 8 pmol of each degenerate primer, 1 mM dNTPs, 2.5 U Taq polymerase (Gibco BRL), 1.5 mM MgCl2, and the manufacturer’s supplied buffer. Following 95°C heating for 5 minutes, protocols of 5 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute, and extension at 74°C for 1 minute followed by 25 cycles of denaturation, annealing at 50°C for 1 minute, and extension were performed. Degenerate primer mixtures were deduced from amino acid sequences PLFDRVL and DILGKYVD and contained Not1 linker sequences: 5’-CGG GGC GGC CGC CCN TT(A/G) TT(T/C) GA(T/C) AG(A/G) GTN TT(A/G) GT-3’ and 5’-GCC CGC GGC CGC TCN AC(A/G) TA(C/T) TTN CCN AG(A/C/T) AT(A/G) TC-3’. These primers produced a PCR product of 282 bp.

Northern and Western blot analysis and in situ hybridization

Total RNA was extracted from zebrafish embryos using Trizol reagent (Life Technologies). Twenty micrograms of total RNA from each sample were denatured and subjected to electrophoresis in a formaldehyde agarose gel according to Sambrook et al (1989). Northern blots were prepared and hybridized with a 282-bp zebrafish cpn10 cDNA fragment labeled with 32P using the random hexamer labeling procedure. After hybridization, Northern blots were subsequently stripped and reprobed with the zebrafish β-actin gene, which provides a suitable control for equal loading and RNA integrity (Pearson et al 1996). RNA integrity and equal loading were determined in Figure 1 by ethidium bromide staining of the gel because β-actin mRNA levels vary considerably throughout early zebrafish development, particularly at pregastrula stages. Effective transfer was determined by visualizing the gel after transfer is complete.

Protein isolation and Western blots were performed according to Sambrook et al (1989) using a rabbit anti-Cpn10 primary antibody (catalog no. SPA-110D, StressGen, Vancouver), a goat anti-rabbit antibody HRP conjugate (BioRad), and chemiluminescence detection (Pierce).

Embryo fixation and in situ hybridization were performed as described in Akimenko et al (1994) with modifications as described in Sass et al (1996).

RESULTS

Cloning of cpn10 in zebrafish

Most studies on Cpn10 have focused on its roles in protein folding and the functional significance of extracellular Cpn10 protein in the serum of mammals. We wished to exploit the advantages of the zebrafish model system to investigate cpn10 gene expression during early vertebrate development. Using degenerate primer RT-PCR, we isolated a cDNA putatively encoding for the zebrafish Cpn10 protein (GenBank accession no. AF273739). We have also isolated zebrafish genomic sequences that contain the cpn10 gene (not shown). These genomic sequences were used to produce a conceptual translation product and used to identify protein coding sequence 5’ to the originally obtained PCR fragment. This produced a near complete coding sequence for cpn10 that lacks only the sequence encoding 3 amino acids residues at the c-terminus. Sequence analysis and alignment, using ClustalV (Higgins et al 1991), of this cDNA indicated that it encodes a protein highly similar to Cpn10 from other vertebrates and prokaryotes (not shown). The overall amino acid sequence identity of our clone compared to the mouse Cpn10 (GenBank accession no. U09659) is 82% and 80% with chicken Cpn10 (GenBank accession no. AF031309).

Embryonic expression of cpn10 in zebrafish

In order to determine if cpn10 is expressed in a developmentally stage-specific manner, we performed North-
Fig 1. Cpn10 is expressed throughout zebrafish development. (A) Northern blot of 1 hour, blastula (4 hours), gastrula (6 hours), midsomitogenesis stage (12 hours), postsomitogenesis (24 hours), and hatching stage (48 hours) zebrafish embryo total RNA (20 µg) hybridized to the zebrafish cpn10 cDNA (upper). The gel was stained with ethidium bromide (lower) prior to transfer to membrane. (B) Western blot of protein extracts from 1 hour, blastula (4 hours), somite stage (12 hours), and 24-hour larval stage zebrafish embryos using an anti-Cpn10 antibody (catalog no. SPA-110D, StressGen, Vancouver).

Fig 2. Northern blot of total RNA isolated from an embryonic derived cell line, ZF4, and 2 adult fin derived cell lines, LFF and AB9, grown at 28°C and hybridized to the zebrafish cpn10 cDNA.

Fig 3. Expression of cpn10 in zebrafish embryos. Whole mount in situ hybridization of 64-cell stage (2 hours), 6-hour gastrula, 24-hour zebrafish embryos (24-hour control), and 24-hour embryos exposed to heat shock (HS) with a zebrafish cpn10 RNA probe.

ern blot analysis on total RNA collected at 1 hour, at the blastula (4 hour), midsomitogenesis (12 hours), postsomitogenesis (24 hours), and hatching stage (48 hours) using the cpn10 cDNA as a probe (Fig 1A). With the exception of RNA samples from 4-hour embryos, the cpn10 mRNA levels are relatively uniform throughout development, although a number of replicates indicated a relatively higher abundance of cpn10 mRNA in 1-hour embryos. Since embryonic transcription in zebrafish does not start until the 1000-cell stage after 4 hours of development (midblastula transition [MBT]), the presence of cpn10 mRNA in 1-hour embryos demonstrates that these cpn10 transcripts are of maternal origin. We do not know if this cpn10 mRNA observed in the zebrafish egg originated from support cells or transcription in the oocyte progenitors cells. The lower levels of cpn10 mRNA in 4-hour embryos indicates that the maternal cpn10 message has been degraded by 4 hours of development, and no other cpn10 transcription is initiated until after MBT. Two transcripts of approximately 1.0 and 1.5 kb were observed in all samples. These 2 transcripts were observed in both total RNA from zebrafish embryos (Fig 1A) and RNA isolated from 3 different zebrafish cell lines (Fig 2). At this point, we do not know the significance of the 2 dif-
different \textit{cpn10} transcripts. Ryan et al (1997) previously noted a variable-length 5’ untranslated region in rat \textit{cpn10} transcripts from adult tissues. However, there appears to be a correlation between developmental stage and the occurrence of the 1.5-kb transcript. The 1.5-kb transcript is almost completely absent relative to the 1.0-kb transcript in total RNA from 1-hour embryos, while higher levels of the 1.5-kb transcript are found in later stages. Similarly, we observed relatively low levels of the 1.5-kb transcript in the cell line ZF4, which is derived from an early zebrafish embryo, compared to 2 other cell lines, derived from adult fin tissue, LFF and AB9, which showed relatively equal amounts of both the 1.0- and the 1.5-kb transcript. The occurrence of the 1.5-kb \textit{cpn10} transcript in embryos and cells that have proceeded past MBT suggests that this transcript is specific to transcription to the zygotic genome and may be associated with more differentiated cell types.

Since gene regulation can also occur at the level of mRNA translation, we performed Western blot analysis using a polyclonal anti-Cpn10 antibody on protein isolated from zebrafish embryos of different developmental stages. Western blot analysis of protein extracts from zebrafish embryos indicates a uniform level of Cpn10 protein in embryos of all developmental stages tested (Fig 1B). We observed a single reactive protein band in all samples tested.

We have shown previously that a number of heat shock protein genes, including \textit{hsp47} and \textit{hsp90\alpha}, are expressed in a tissue-specific manner during normal embryonic development of the zebrafish, while \textit{hsp70} shows tissue-specific expression following exposure to heat shock (Lele and Krone 1997; Lele et al 1997; Sass and Krone 1997). To examine if \textit{cpn10} transcription in zebrafish is heat inducible, mRNA and protein were isolated from 48-hour zebrafish larvae raised at 27°C and larvae exposed to an elevated water temperature of 37°C for up to 4 hours (Fig 4). \textit{Cpn10} mRNA levels peaked after 1 hour of heat shock with a subsequent overall reduction in the level of \textit{cpn10} mRNA. Using Northern and Western blot analysis, we detected substantially higher levels of both \textit{cpn10} mRNA and protein from embryos subjected to heat shock (37°C) for 1 hour compared to embryos maintained at 27°C (Fig 5A,B). This peak of transcriptional induction of \textit{cpn10} by heat shock is short-lived as indicated by a relatively significant drop in \textit{cpn10} mRNA after only 1 hour of recovery at 27°C. The level of \textit{cpn10} mRNA returns to basal levels after 4 hours of recovery. Levels of Cpn10 protein remain elevated relative to levels found in control embryos even at 4 hours of recovery, indicating a greater stability of the Cpn10 protein than \textit{cpn10} mRNA.

**Early pregnancy factor is not found in serum of gravid female zebrafish**

Cpn10 protein is found in the circulating serum of female mammals shortly following fertilization of an egg (Morton et al 1987). It has been suggested that this circulating Cpn10 causes suppression of the female immune system and thus prevents an immune attack on the embryo as it implants in the uterus (Rolfe et al 1988). The zebrafish provides an excellent model system to test this hypothesis because its embryos develop externally, and thus we would not expect to find high circulating levels of Cpn10 in adult zebrafish.

In order to determine if Cpn10 might be involved in nonimmunosuppression roles, we collected serum from male zebrafish and gravid female zebrafish. Gravid females are females that contain oocytes at various stages of development (Selman et al 1993) and are spawning regularly. Western blot analysis of serum from a number of both male and female fish revealed the complete absence of Cpn10 protein in the blood serum fractions (Fig 6) even when relatively large amounts of protein were transferred to Western blots and/or when films were exposed to the HRP-chemiluminescence reaction for long periods of time.

**DISCUSSION**

Previous work in our laboratory studying the expression of some heat shock protein genes during zebrafish embryogenesis has suggested that these proteins, which

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**Hsp 10 expression is induced in zebrafish on exposure to heat shock**

Most heat shock protein genes of eukaryotes exhibit a substantial upregulation of transcription, called the heat shock response, when cells are exposed to environmental stress or heat shock. Indeed, we have observed previously that the zebrafish heat shock genes \textit{hsp47}, \textit{hsp70}, and \textit{hsp90\alpha} are heat-inducible (Lele et al 1997; Sass and Krone 1997). To examine if \textit{cpn10} transcription in zebrafish is heat inducible, mRNA and protein were isolated from 48-hour zebrafish larvae raised at 27°C and larvae exposed to an elevated water temperature of 37°C for up to 4 hours (Fig 4). \textit{Cpn10} mRNA levels peaked after 1 hour of heat shock with a subsequent overall reduction in the level of \textit{cpn10} mRNA. Using Northern and Western blot analysis, we detected substantially higher levels of both \textit{cpn10} mRNA and protein from embryos subjected to heat shock (37°C) for 1 hour compared to embryos maintained at 27°C (Fig 5A,B). This peak of transcriptional induction of \textit{cpn10} by heat shock is short-lived as indicated by a relatively significant drop in \textit{cpn10} mRNA after only 1 hour of recovery at 27°C. The level of \textit{cpn10} mRNA returns to basal levels after 4 hours of recovery. Levels of Cpn10 protein remain elevated relative to levels found in control embryos even at 4 hours of recovery, indicating a greater stability of the Cpn10 protein than \textit{cpn10} mRNA.

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**DISCUSSION**

Previous work in our laboratory studying the expression of some heat shock protein genes during zebrafish embryogenesis has suggested that these proteins, which
have in the past been assigned primarily ‘housekeeping’ roles, may be more directly involved in embryonic cellular differentiation. 

**Hsp47**, for example, was found to be coexpressed with type II collagen in a tissue-specific manner in the developing zebrafish notochord (Lele and Krone 1997). Moreover, the **hsp90a** gene of zebrafish is expressed in a spatial and temporal manner consistent with a role in the differentiation of slow and fast muscle lineages during development (Sass et al 1996, 1999). This hypothesis is supported by the fact that pharmacological inhibition of Hsp90 results in abnormal muscle development and somite patterning (Lele et al 1999).

Our analysis of the expression of **cpn10** revealed no tissue-specific expression during zebrafish development. This uniform and ubiquitous expression of **cpn10** throughout zebrafish development (Figs 1 and 3) suggests that Cpn10 plays a general cellular function. Since assays for tissue- and developmental stage-specific expression of **cpn10** have not been conducted on the other vertebrate species for which **cpn10** has been cloned, we are unable to make any conclusions on the conservation of **cpn10** expression during embryogenesis. Zygotic gene transcription does not occur in the zebrafish until the 1000-cell stage (approximately 4 hours postfertilization); thus, the relative high level of **cpn10** mRNA found in 1-hour embryos is of maternal origin. This maternal reservoir of **cpn10** mRNA is likely required to maintain the uniform levels of Cpn10 protein found during early development prior to the activation of the zygotic genome.

The importance of heat shock proteins in the folding and transport of proteins is reflected in their expression during normal embryo development (Morimoto et al 1994). The expression characteristics of **cpn10** of zebrafish are consistent with Cpn10 chaperone functions in the mitochondria of every cell. Since conditions of cellular stress (heat shock) lead to the accumulation of denatured and aberrantly folded proteins, the induction of **cpn10** transcription (Figs 4 and 5) during heat shock implies a functional role in aiding in the proper assembly and folding of proteins during times of cellular stress in zebrafish embryos.

The zebrafish embryo develops at an extremely fast rate compared to most other vertebrate embryos—going from a 1 cell embryo to a larva in 48 hours. It has been suggested that extracellular Cpn10 (EPF) found in pregnant mammal serum might act as a growth factor (Quinn et al 1990). While we cannot discount the idea that Cpn10 may play a growth factor role in the zebrafish embryo, it is more likely that Cpn10 is primarily involved in protein folding in the mitochondria, given the high energetic requirements of the rapidly developing embryo. It is intriguing that we observe a single maternal transcript of approximately 1 kb in early embryos and a cell line derived from early embryos while observing an addition.
transcript of larger molecular weight in older embryos and differentiated cell cultures. This observation might suggest an alternate transcription start site associated with zygotic transcription and potentially indicate specific functions for Cpn10 in the early embryo.

There is considerable support that EPF in pregnant mammal serum acts to suppress the mother’s immune system, thus preventing a negative immune response against the implanting embryo (Rolfe et al 1988). The complete absence of Cpn10 protein in the serum of female zebrafish is not surprising since zebrafish are oviparous, and therefore we would not expect there to be a need to suppress the immune system of gravid female zebrafish. Although this does not definitively support the immunosuppressive role of EPF in mammals, it suggests that the secreted form of Cpn10 may be a novel acquisition of mammalian vertebrates.

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